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# Patents Form 1/77

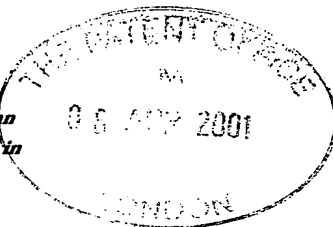
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PRO1 E620347-1 D01298  
101/7700 0.00-0108730.3

## Request for grant of a patent

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1. Your reference

PCS22013FAE-PROV

2. Patent application number

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**0108730.3**

3. Full name, address and postcode of the or of each applicant (*underline all surnames*)

PFIZER LIMITED  
RAMSGATE ROAD  
SANDWICH  
KENT CT13 9NJ

Patents ADP number (*if you know it*)

If the applicant is a corporate body, give the country/state of its incorporation

UNITED KINGDOM

068912673001

4. Title of the invention

TREATMENT OF MALE SEXUAL DYSFUNCTION

5. Name of your agent (*if you have one*)

DR. F.A. EDWARDS

"Address for service" in the United Kingdom to which all correspondence should be sent (*including the postcode*)

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Patents ADP number (*if you know it*)

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6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and (*if you know it*) the or each application number

Country

Priority application number  
(*if you know it*)

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7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application

Date of filing  
(*day / month / year*)

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? (*Answer 'Yes' if:*

- a) any applicant named in part 3 is not an inventor, or
  - b) there is an inventor who is not named as an applicant, or
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Description	153	/
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### Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (*Patents Form 7/77*)

Request for preliminary examination and search (*Patents Form 9/77*)

Request for substantive examination (*Patents Form 10/77*)

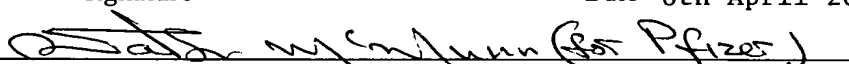
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Annexe 1

11. I/We request the grant of a patent on the basis of this application.

Signature

Date 6th April 2001

 (for Pfizer)

12. Name and daytime telephone number of person to contact in the United Kingdom  
Dr. F.A. Edwards 01304.641687

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## TREATMENT OF MALE SEXUAL DYSFUNCTION

### FIELD OF INVENTION

5 The present invention relates to compounds and pharmaceutical compositions for use in the treatment of male sexual dysfunction, in particular male erectile dysfunction (MED).

The present invention also relates to a method of treatment of MED.

10

The present invention also relates to assays to screen for the compounds of the present invention and which form part of the pharmaceutical compositions of the present invention and which are useful in the treatment of male sexual dysfunction, in particular MED.

15

For convenience, a list of abbreviations that are used in the following text is presented before the Claims section.

### SEXUAL DYSFUNCTION

20

Sexual dysfunction (SD) is a significant clinical problem which can affect both males and females. The causes of SD may be both organic as well as psychological. Organic aspects of SD are typically caused by underlying vascular diseases, such as those associated with hypertension or diabetes mellitus, by prescription medication and/or by psychiatric disease such as depression. Physiological factors include fear, performance anxiety and interpersonal conflict. SD impairs sexual performance, diminishes self-esteem and disrupts personal relationships thereby inducing personal distress. In the clinic, SD disorders have been divided into female sexual dysfunction (FSD) disorders and male sexual dysfunction (MSD) disorders (Melman *et al* 1999).

30

FSD is best defined as the difficulty or inability of a woman to find satisfaction in sexual expression. Male sexual dysfunction (MSD) is generally associated with erectile dysfunction, also known as male erectile dysfunction (MED) (Benet *et al* 1994).

35

## MALE ERECTILE DYSFUNCTION (MED)

It is known that some individuals can suffer from male erectile dysfunction (MED).

5 Male erectile dysfunction (MED) is defined as:

“the inability to achieve and/or maintain a penile erection for satisfactory sexual performance” (NIH Consensus Development Panel on Impotence, 1993)”

10 It has been estimated that the prevalence of erectile dysfunction (ED) of all degrees (minimal, moderate and complete impotence) is 52% in men 40 to 70 years old, with higher rates in those older than 70 (Melman *et al* 1999). The condition has a significant negative impact on the quality of life of the patient and their partner, often resulting in increased anxiety and tension which leads to depression and low self  
15 esteem. Whereas two decades ago, MED was primarily considered to be a psychological disorder (Benet *et al* 1994), it is now known that for the majority of patients there is an underlying organic cause. As a result, much progress has been made in identifying the mechanism of normal penile erection and the pathophysiology of MED.

20

Penile erection is a haemodynamic event which is dependent upon the balance of contraction and relaxation of the corpus cavernosal smooth muscle and vasculature of the penis (Lerner *et al* 1993). Corpus cavernosal smooth muscle is also referred to herein as corporal smooth muscle or in the plural sense corpus cavernosa.

5 Relaxation of the corpus cavernosal smooth muscle leads to an increased blood flow into the trabecular spaces of the corpus cavernosa, causing them to expand against the surrounding tunica and compress the draining veins. This produces a vast elevation in blood pressure which results in an erection (Naylor, 1998).

30 The changes that occur during the erectile process are complex and require a high degree of co-ordinated control involving the peripheral and central nervous systems, and the endocrine system (Naylor, 1998). Corporal smooth muscle contraction is modulated by sympathetic noradrenergic innervation via activation of postsynaptic  $\alpha_1$  adrenoceptors. MED may be associated with an increase in the endogenous smooth  
35 muscle tone of the corpus cavernosum. However, the process of corporal smooth muscle relaxation is mediated primarily by non-adrenergic, non-cholinergic (NANC) neurotransmission. There are a number of other NANC neurotransmitters found in

the penis, other than NO, such as calcitonin gene related peptide (CGRP) and vasoactive intestinal peptide (VIP). The main relaxing factor responsible for mediating this relaxation is nitric oxide (NO), which is synthesised from L-arginine by nitric oxide synthase (NOS) (Taub *et al* 1993; Chuang *et al* 1998). It is thought that reducing corporal smooth muscle tone may aid NO to induce relaxation of the corpus cavernosum. During sexual arousal in the male, NO is released from neurones and the endothelium and binds to and activates soluble guanylate cyclase (sGC) located in the smooth muscle cells and endothelium, leading to an elevation in intracellular cyclic guanosine 3',5'-monophosphate (cGMP) levels. This rise in cGMP leads to a relaxation of the corpus cavernosum due to a reduction in the intracellular calcium concentration ( $[Ca^{2+}]_i$ ), via unknown mechanisms thought to involve protein kinase G activation (possibly due to activation of  $Ca^{2+}$  pumps and  $Ca^{2+}$ -activated  $K^+$  channels; Chuang *et al.*, 1998).

MED mainly arises from an inability of NO released during sexual arousal to effectively relax corpus cavernosal smooth muscle. It has therefore been proposed that MED may be treatable by potentiating or facilitating nitrgenic signalling thereby leading to an elevation in intracellular cGMP levels. Nitrgenic signalling as defined herein means the cellular mechanisms that are activated by the NO released or generated during sexual arousal / stimulation and in particular relates to the activation of the guananyl cyclase / cGMP pathway. In this respect, sildenafil citrate (also known as Viagra<sup>TM</sup>) has recently been developed by Pfizer as the first oral drug treatment for MED. Sildenafil acts by inhibiting cGMP breakdown in the corpus cavernosa by selectively inhibiting phosphodiesterase 5 (PDE5), thereby limiting the hydrolysis of cGMP to 5'GMP (Boolel *et al.*, 1996; Jeremy *et al.*, 1997) and thereby increasing the intracellular concentrations of cGMP and facilitating corpus cavernosal smooth muscle relaxation.

Currently, all other available MED therapies on the market, such as treatment with prostaglandin based compounds i.e. alprostadil which can be administered intra-urethrally (available from Vivus Inc., as Muse<sup>TM</sup>) or via small needle injection (available from Pharamcia & Upjohn, as Caverject<sup>TM</sup>), are either inconvenient and/or invasive. Other treatments include vacuum constriction devices, vasoactive drug injection or penile prostheses implantation (Montague *et al.*, 1996). Although injectable vasoactive drugs show high efficacy, side effects such as penile pain, fibrosis and priapism are common, and injection therapy is not as convenient as oral

therapy therefore sildenafil currently represents the most preferred therapy on the market.

5 The present invention relates to compounds and pharmaceutical compositions useful for the treatment of MED.

### SUMMARY ASPECTS OF THE PRESENT INVENTION

10 A seminal finding of the present invention is the ability to treat a male suffering from MED with use of an I:NPY.

In accordance with the present invention, the I:NPY of the present invention is referred to as the "agent of the present invention". The terms I:NPY and NPYi are  
15 used interchangeably hereinafter.

As defined herein, an I:NPY is an entity (i.e. a biological material or a chemical compound) which inhibits the action of NPY, such as the biological action of NPY within the human body. An entity which inhibits the biological action of NPY, can be  
20 defined as, an entity which can interfere with the formation of NPY, and/or interfere with the breakdown of NPY (i.e. metabolism), and/or inhibit the ability of any (or all) of the NPY receptors to respond to the influence or action of NPY or other entities. NPY receptors are as defined hereinafter.

5 Highly preferred for use herein in the treatment of MED are NPY receptor inhibitors and in particular NPY Y1 and/or NPY Y5 receptor inhibitors and especially NPY Y1 receptor inhibitors, most especially selective NPY Y1. NPY receptor inhibitors, NPY Y1 and/or NPY Y5 receptor inhibitors, and NPY Y1 receptor inhibitors are encompassed by the term "an agent of the present invention" as defined  
30 hereinbefore.

A further seminal finding of the present invention is the ability to enhance male genital blood flow with use of the agent of the present invention.

35 It is proposed herein that treatment of men with MED can be achieved by enhancement of genital blood flow with vasoactive agents. In our studies, we have shown that cAMP mediates corpus cavernosal vasorelaxation and that genital blood

flow can be enhanced/potentiated by elevation of cAMP levels. This is a further seminal finding.

Thus, in a broad aspect, the present invention relates to the use of a cAMP potentiator to treat MED.

According to the present invention, the target is a  $P_{\text{cAMP}}$  target, which  $P_{\text{cAMP}}$  target is an inhibitor of NPY or one of its associated receptors.

10 Nucleotide sequences and amino acid sequences for NPY and its receptors are available in the literature. Some sequences are presented in the Sequence Listings provided herein.

15 Here, we have found that inhibitors of neuropeptide Y receptors (i.e. antagonists or the NPY receptors) can increase intercavernosal pressure. Thus, we have demonstrated that NPY receptors play a role in influencing the blood flow process in male genitalia. Hence, a suitable target for the treatment of MED is NPY or one of its associated receptors.

20 Thus, in one preferred aspect, the agent is an NPY  $Y_1$   $Y_2$  or  $Y_5$  antagonist, preferably an oral NPY  $Y_1$   $Y_2$  or  $Y_5$  antagonist. This agent will treat MED by increasing intercavernosal (i.c.) pressure such as for example via influencing genital blood flow.

25 Thus NPY inhibitors increases i.c. pressure and therefore NPY represents a potential therapeutic target by which blood flow in the male genitalia can be influenced. The mechanism through which this antagonism occurs is most likely through NPY  $Y_1$  receptor-induced  $G_{i/o}$  activation. In other physiological systems NPY  $Y_1$  receptors have been implicated in mediating vasoconstriction and inhibiting sympathetic transmitter release (Lundberg *et al.*, 1996; a NPY  $Y_2$  effect can not be excluded).

30

As indicated, a  $P_{\text{cAMP}}$  target is one of the NPY receptors.

35 The present invention is advantageous as it provides a means for restoring a normal sexual arousal response - namely increased genital blood flow. Hence, the present invention provides a means to restore, or potentiate, the normal sexual arousal response.

In accordance with the present invention, the agent of the present invention acts on a target, preferably specifically on that target. This target is sometimes referred to as the "target of the present invention". However, the agent of the present invention may act on one or more other targets. These other targets may be referred to as an "additional target". Likewise, if an additional agent is used, then that additional agent can target the same target of the present invention and/or an additional target (which need not be the same additional target that is acted on by the agent of the present invention). Targets are described herein. It is to be understood that general references herein to targets may be applicable to the additional targets as well as to the target of the present invention.

#### DETAILED ASPECTS OF THE PRESENT INVENTION

In one aspect, the present invention relates to a pharmaceutical composition for use (or when in use) in the treatment of MED; the pharmaceutical composition comprising an agent capable of potentiating cAMP in the sexual genitalia of a male suffering from MED; wherein the agent is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient; and wherein said agent is the agent of the present invention as herein defined. Here, the composition (like any of the other compositions mentioned herein) may be packaged for subsequent use in the treatment of MED.

In another aspect, the present invention relates to the use of an agent in the manufacture of a medicament (such as a pharmaceutical composition) for the treatment of MED; wherein the agent is capable of potentiating cAMP in the sexual genitalia of a male suffering from MED; and wherein said agent is the agent of the present invention as herein defined.

In a further aspect, the present invention relates to a method of treating a male suffering from MED; the method comprising delivering to the male an agent that is capable of potentiating cAMP in the sexual genitalia; wherein the agent is in an amount to cause potentiation of cAMP in the sexual genitalia of the male; wherein the agent is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient; and wherein said agent is the agent of the present invention as herein defined.

In a further aspect, the present invention relates to an assay method for identifying an agent that can be used to treat MED, the assay method comprising: determining whether an agent can directly or indirectly potentiate cAMP; wherein a potentiation of cAMP in the presence of the agent is indicative that the agent may be useful in the treatment of MED; and wherein said agent is an I:NPY.

By way of example, the present invention relates to an assay method for identifying an agent that can directly or indirectly potentiate cAMP in order to treat MED, the assay method comprising: contacting an agent with a moiety capable of affecting cAMP activity and/or levels; and measuring the activity and/or levels of cAMP; wherein a potentiation of cAMP in the presence of the agent is indicative that the agent may be useful in the treatment of MED; and wherein said agent is an I:NPY.

By way of further example, the present invention relates to an assay method for identifying an agent that can directly or indirectly potentiate cAMP in order to treat MED, the assay method comprising: contacting an agent with cAMP; and measuring the activity of cAMP; wherein a potentiation of cAMP in the presence of the agent is indicative that the agent may be useful in the treatment of MED; and wherein said agent is I:NPY.

In a further aspect, the present invention relates to a process comprising the steps of: (a) performing the assay according to the present invention; (b) identifying one or more agents that can directly or indirectly potentiate cAMP activity; and (c) preparing a quantity of those one or more identified agents; and wherein said agent is an I:NPY.

With this aspect, the agent identified in step (b) may be modified so as to, for example, maximise activity and then step (a) may be repeated. These steps may be repeated until the desired activity or pharmacokinetic profile has been achieved.

Thus, in a further aspect, the present invention relates to a process comprising the steps of: (a1) performing the assay according to the present invention; (b1) identifying one or more agents that can directly or indirectly potentiate cAMP activity, (b2) modifying one or more of said identified agents; (a2) optionally repeating step (a1); and (c) preparing a quantity of those one or more identified agents (i.e. those that have been modified); and wherein said agent is an I:NPY.

In a further aspect, the present invention relates to a method of treating MED, by potentiating *in vivo* cAMP with an agent; wherein the agent is capable of directly or indirectly potentiating cAMP in an *in vitro* assay method; wherein the *in vitro* assay method is the assay method according to the present invention; and wherein said agent is an I:NPY.

In a further aspect, the present invention relates to the use of an agent in the preparation of a pharmaceutical composition for the treatment of MED, wherein the agent is capable of directly or indirectly potentiating cAMP when assayed *in vitro* by the assay method according to the present invention; and wherein said agent is an I:NPY.

In one aspect, the present invention relates to NPYi compounds and pharmaceutical compositions including NPYi compounds and pharmaceutical combinations comprising NPYi and PDE5i for use (or when in use) in the treatment of male sexual dysfunction, in particular MED. In said pharmaceutical compositions the NPYi (and PDE5i, if present, and/or additional agent) is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient. Here, the composition (like any of the other compositions mentioned herein) may be packaged for subsequent use in the treatment of male sexual dysfunction, in particular MED.

In another aspect, the present invention relates to the use of an agent in the manufacture of a medicament (such as a pharmaceutical composition) for the treatment of male sexual dysfunction, in particular MED.

In a further aspect, the present invention relates to a method of treating a male suffering from male sexual dysfunction, in particular MED; the method comprising delivering to the male an NPYi that is capable of enhancing the endogenous erectile process in the corpus cavernosum; wherein the NPYi is present in an amount to enhance the endogenous erectile process as defined hereinbefore; wherein the NPYi is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient; and wherein said NPYi is as herein defined.

In a further aspect, the present invention relates to an assay method for identifying an agent (hereinafter referred to as a NPYi) that can be used to treat male sexual dysfunction, in particular MED, the assay method comprising: determining whether a test agent can directly enhance the endogenous erectile process; wherein said enhancement is defined as a potentiation of intracavernosal pressure (ICP) (and/or



cavernosal blood flow) in the presence of a test agent as defined herein; such potentiation by a test agent is indicative that the test agent may be useful in the treatment of male sexual dysfunction, in particular MED and wherein said test agent is a NPYi.

5

In a further aspect, the present invention relates to an animal model used to identify agents capable of treating MED, said model comprising an anaesthetised male animal including means to measure changes in genital blood flow of said animal following stimulation of the pelvic nerve thereof; and wherein said agent is an I:NPY.

10

In a further aspect, the present invention relates to an assay method for identifying an agent that can directly or indirectly potentiate cAMP in order to treat MED, the assay method comprising: administering an agent to the animal model of the present invention; and measuring any potentiation of cAMP and/or increase in blood flow in the genitalia of said male animal; and wherein said agent is an I:NPY.

15

In a further aspect, the present invention relates to a diagnostic method, the method comprising isolating a sample from a male; determining whether the sample contains an entity present in such an amount to cause MED, or is in an amount so as to cause MED; wherein the entity has a direct or indirect effect on the level or activity of cAMP in the sexual genitalia of the male; and wherein said entity can be modulated to achieve a beneficial effect by use of an agent; and wherein said agent is an I:NPY.

20

In a further aspect, the present invention relates to a diagnostic composition or kit comprising means for detecting an entity in an isolated male sample; wherein the means can be used to determine whether the sample contains the entity and in such an amount to cause MED, or is in an amount so as to cause MED; wherein the entity has a direct or indirect effect on the level or activity of cAMP in the sexual genitalia of the male and wherein said entity can be modulated to achieve a beneficial effect by use of an agent; and wherein said agent is an I:NPY.

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According to a further embodiment the present invention provides the use of one or more NPYi compounds in combination with one or more PDE5i's for the treatment of MED.

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Preferably said combined treatment comprises a combination of one or more NPYi compounds with one or more PDE5i's. More preferably such combination provides for the concomitant administration of one or more NPYi compounds with one or more PDE5i's for the treatment of MED.

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Highly preferred herein is the use of a pharmaceutical composition comprising one or more NPYi compounds with one or more PDE5i's for the treatment of MED.

Especially preferred for use in the pharmaceutical compositions for the treatment of MED according to the present invention is the combination of a potent and selective NPYi compound with a potent and selective PDE5i.

In a preferred embodiment herein said combined administration of NPYi compound and PDE5i is concomitant. Concomitant administration as defined herein encompasses simultaneous (separate) administration, simultaneous combined administration, separate administration, combined administration, sequential administration and co-formulated combined administration of a cGMP PDE5i and a NPYi compound.

Thus, it is a further object of the present invention to provide pharmaceutical compositions comprising an NPYi compound and a PDE5i for use in the treatment of MED.

For ease of reference, these and further aspects of the present invention are now discussed under appropriate section headings. However, the teachings under each section are not necessarily limited to each particular section.

General references herein to agents may be applicable to "auxiliary" additional agents as well as to agents of the present invention.

For some applications, preferably the additional agent has an indirect potentiating effect on cAMP. Examples of such additional agents include I:NEP and/or I:NPY. Alternatively expressed, for some applications, preferably the additional agent does not have a direct potentiating effect on cAMP. It is to be understood that the additional agent may have an indirect potentiating effect on cAMP by acting on

naturally found and naturally located directly acting agents – such as naturally found and located VIP.

For some applications, preferably the additional agent has a direct potentiating effect on cAMP. Examples of such additional agents include I:PDE.

For some applications, the additional agent is an inhibitor – i.e. it is capable of exhibiting an inhibitory function.

For some applications, the additional agent is an antagonist.

For some applications, preferably the additional agent is an I:PDE (sometimes written as PDEi)

For some applications, preferably the additional agent is a selective I:PDE.

For some applications, preferably the additional agent is an I:PDE1 or I:PDE2 (sometimes written as I:PDE1i or PDE1i or PDE2i) or I:PDE3 or I:PDE4 or I:PDE7 or I:PDE8, more preferably the agent is an I:PDE2.

For some applications, preferably the additional agent is a selective I:PDE1i.

For some applications, preferably the additional agent is a I:NEP (sometimes written as NEPi).

For some applications, preferably the additional agent is a selective I:NEP.

For some applications, preferably the additional agent is a I:NEP wherein said NEP is EC 3.4.24.11.

For some applications, preferably the additional agent is a selective I:NEP wherein said NEP is EC 3.4.24.11.

In accordance with the use of NPYi compounds for the treatment of MED according to the as discussed hereinbefore, the NPYi acts on a target, preferably specifically on that target. For example where a combination of a NPYi and a PDE5i are present the targets are the NPY and PDE5 enzymes. This target is sometimes referred to as

the "target of the present invention". However, the additional agents of the present invention may act on one or more other targets. These other targets may be referred to as an "additional target". Likewise, if an additional agent is used, then that additional agent can target the same target of the present invention and/or an additional target (which need not be the same additional target that is acted on by the agent of the present invention). Targets are described herein. It is to be understood that general references herein to targets may be applicable to the additional targets as well as to the target of the present invention.

## PREFERABLE ASPECTS

### NPY Inhibitors

The agents for use in the treatment of MED according to the present invention are NPY inhibitors.

In one embodiment, preferably the agent for the use according to the present invention may be used via oral administration.

For some applications, preferably the agent for the use according to the present invention is a selective NPYi.

Preferably, the agent of the present invention is a mediator of male genital vasorelaxation.

Preferably the agent of the present invention is an antagonist.

The agent of the present invention is an I:NPY (sometimes written as NPYi).

For some applications, preferably the agent is an I:NPY Y1 or I:NPY Y2 or I:NPY Y5, more preferably the agent is an I:NPY Y1.

Preferably the agent is a selective I:NPY.

Preferably the agent is an I:NPY Y1.

Preferably the agent is a selective I:NPY Y1.

As indicated above, the agent may be any suitable agent that can act as an I:NPY  
5 (sometimes referred to as an NPY antagonist).

Background teachings on NPY and its associated receptors have been prepared by  
Victor A. McKusick et al on <http://www3.ncbi.nlm.nih.gov/Omim/searchomim.htm>.  
The following text concerning NPY has been extracted from that source.

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"Neuropeptide Y (NPY) is an abundant and widespread peptide in the mammalian nervous system. It shows sequence homology to peptide YY and over 50% homology in amino acid and nucleotide sequence to pancreatic polypeptide (PNP; 167780). NPY is a 36-amino acid peptide. Minth et al. (1984) cloned the NPY gene starting from mRNA of a pheochromocytoma. Takeuchi et al. (1985, 1986) isolated cDNA clones of the NPY and PNP genes from a pheochromocytoma and a pancreatic endocrine tumor, respectively. Using these cDNA probes to analyze genomic DNA from chromosome assignment panels of human-mouse somatic cell hybrids, they then examined the question of whether the genes are syntenic. The studies showed nonsynteny, with NPY on 7pter-7q22 and PNP on 17p11.1-17qter. By studies of a backcross with *Mus spretus*, Bahary et al. (1991) mapped the homologous NPY gene to mouse chromosome 6. Since mouse chromosome 6 has homology to human 7q, it is likely that the NPY gene in man is located in the region 7cen-q22. Meisler et al. (1987) excluded close linkage between the loci for cystic fibrosis (219700) and neuropeptide Y. Terenghi et al. (1987) determined the distribution of mRNA encoding NPY in neurons of the cerebral cortex in surgical biopsy specimens and postmortem brain by means of in situ hybridization techniques. They showed consistent localization of NPY gene transcription and expression in normal mature cortical neurons. Baker et al. (1995) showed by fluorescence in situ hybridization that the NPY gene is located on 7p15.1 and exists in single copy. They commented that NPY is one of the most highly conserved peptides known, with, for example, only 3 amino acid differences between human and shark. Neuropeptide Y is a neuromodulator implicated in the control of energy balance and is overproduced in the hypothalamus of ob/ob mice. To determine the role of NPY in the response to leptin (164160) deficiency, Erickson et al. (1996) generated ob/ob mice deficient in NPY. In the absence of NPY, ob/ob mice were less obese because of reduced food intake and increased energy expenditure, and were less severely affected by diabetes, sterility, and somatotrophic defects. These results were interpreted as indicating that NPY is a central effector of leptin deficiency. Genetic linkage analysis

of rats that were selectively bred for alcohol preference identified a chromosomal region that included the NPY gene (Carr et al., 1998). Alcohol-preferring rats had lower levels of NPY in several brain regions compared with alcohol-nonpreferring rats. Thiele et al. (1998) therefore studied alcohol consumption by mice that completely lacked NPY as a result of targeted gene disruption (Erickson et al., 1996). They found that NPY-deficient mice showed increased consumption, compared with wildtype mice, of solutions containing 6%, 10%, and 20% (by volume) ethanol. NPY-deficient mice were also less sensitive to the sedative/hypnotic effects of ethanol, as shown by more rapid recovery from ethanol-induced sleep, even though plasma ethanol concentrations did not differ significantly from those of controls. In contrast, transgenic mice that overexpressed a labeled NPY gene in neurons that usually express it had a lower preference for ethanol and were more sensitive to the sedative/hypnotic effects of ethanol than controls. These data provided direct evidence that alcohol consumption and resistance are inversely related to NPY levels in the brain. As part of an on-going study of the genetic basis of obesity, Karvonen et al. (1998) identified a 1128T-C polymorphism that resulted in substitution of leucine by proline at residue 7 in the signal peptide part of pre-pro-NPY. This polymorphism was not associated with obesity or energy metabolism, but was significantly and consistently associated with high serum total and LDL cholesterol levels both in normal-weight and obese Finns and in obese Dutch subjects. Uusitupa et al. (1998) found the pro7 polymorphism in 14% of Finns but in only 6% of Dutchmen. Subjects with pro7 in NPY had, on average, 0.6 to 1.4 mmol/L higher serum total cholesterol levels than those without this gene variant. As the impact of pro7 NPY on serum cholesterol levels could not be found in normal-weight Dutchmen, it can be assumed that obese persons may be more susceptible to the effect of the gene variant. It was calculated that the probability of having the pro7 in NPY could be as high as 50 to 60% in obese subjects with a total serum cholesterol equal to or higher than 8 mmol/L. At least among Finns, the pro7 form of NPY is one of the strongest genetic factors affecting serum cholesterol levels. SEE ALSO Allen and Bloom (1986) ; Dockray (1986) ; Maccarrone and Jarrott (1986) ; Minth et al. (1986)."

As indicated background teachings on NPY and its associated receptors have been prepared by Victor A. McKusick et al (ibid). The following text concerning NPYR1 has been extracted from that source.

5 "Neuropeptide Y (NPY; 162640) is one of the most abundant neuropeptides in the mammalian nervous system and exhibits a diverse range of important physiologic activities, including effects on psychomotor activity, food intake, regulation of central endocrine secretion, and potent vasoactive effects on the cardiovascular system. Two major subtypes of NPY (Y1 and Y2) have been defined by pharmacologic  
10 criteria. The NPY Y1 receptors have been identified in a variety of tissues, including brain, spleen, small intestine, kidney, testis, placenta, and aortic smooth muscle. The Y2 receptor is found mainly in the central nervous system. Herzog et al. (1992) reported cloning of a cDNA encoding a human NPY receptor which they confirmed to be a member of the G protein-coupled receptor superfamily. When expressed in  
15 Chinese hamster ovary (CHO) or human embryonic kidney cells, the receptor exhibited characteristic ligand specificity. In the kidney cell line, the receptor was coupled to a pertussis toxin-sensitive G protein that mediated the inhibition of cyclic AMP accumulation. In the CHO cell line, on the other hand, the receptor was coupled not to inhibition of adenylate cyclase but rather to the elevation of intracellular calcium. Thus the second messenger coupling of the NPY receptor was cell type  
20 specific, depending on the specific repertoire of G proteins and effector systems present in the cell type. Larhammar et al. (1992) independently cloned and characterized the neuropeptide Y receptor. Herzog et al. (1993) determined the molecular organization and regulation of the human NPY Y1 receptor gene. In contrast to the contiguous structure of most G protein-coupled receptor genes, they  
25 found that the NPY Y1 receptor gene has 3 exons. They also identified a common PstI polymorphism in the first intron of the gene. By high resolution fluorescence in situ hybridization, they localized the gene to 4q31.3-q32. Herzog et al. (1997) found that the NPY1R and NPY5R (602001) genes are colocalized on chromosome 4q31-q32. The 2 genes are transcribed in opposite directions from a common promoter region. One of the alternately spliced 5-prime exons of the Y1 receptor gene is a part of the coding sequence of the Y5 receptor. This unusual arrangement suggested to Herzog et al. (1997) that the 2 genes arose by a gene duplication event and that they may be coordinately expressed. By interspecific backcross analysis, Lutz et al.  
30 (1997) mapped the Npy1r and Npy2r genes to conserved linkage groups on mouse chromosomes 8 and 3, respectively, which correspond to the distal region of human chromosome 4q."

As indicated background teachings on NPY and its associated receptors has been prepared by Victor A. McKusick et al (ibid). The following text concerning NPYR2 has been extracted from that source.

5 "Neuropeptide Y (NPY) signals through a family of G protein-coupled receptors present in the brain and sympathetic neurons. At least 3 types of neuropeptide Y receptor have been defined on the basis of pharmacologic criteria, tissue distribution, and structure of the encoding gene; see 162641 and 162643. Rose et al. (1995) reported the expression cloning in COS cells of a cDNA for the human type 2  
10 receptor, NPY2R. Transfected cells showed high affinity for NPY (162640), peptide YY (PYY; 600781), and a fragment of NPY including amino acids 13 to 36. The predicted 381-amino acid protein has 7 transmembrane domains characteristic of G protein-coupled receptors and is only 31% identical to the human Y1 receptor (NPY1R; 162641). A 4-kb mRNA was detected on Northern blots of tissue samples  
15 from several regions of the nervous system. Gerald et al. (1995) cloned the cDNA corresponding to the human Y2 receptor from a human hippocampal cDNA expression library using a radiolabeled PYY-binding assay. They expressed the Y2 gene in COS-7 cells and performed a hormone-binding assay which showed that the Y2 receptor binds (from highest to lowest affinity) PYY, NPY, and pancreatic polypeptide (PP; 167780) hormones. Ammar et al. (1996) cloned and characterized the human gene encoding the type 2 NPY receptor. The transcript spans 9 kb of genomic sequence and is encoded in 2 exons. As in the type 1 NPY receptor gene, the 5-prime untranslated region of NPY2R is interrupted by a 4.5-kb intervening sequence. Ammar et al. (1996) demonstrated by Southern analysis of rodent-human  
20 cell hybrids followed by fluorescence in situ hybridization (FISH) that the NPY2R gene maps to 4q31, the same region containing the NPY1R gene, suggesting that these subtypes may have arisen by gene duplication despite their structural differences. By interspecific backcross analysis, Lutz et al. (1997) mapped the Npy1r and Npy2r genes to conserved linkage groups on mouse chromosomes 8 and  
25 3, respectively, which correspond to the distal region of human chromosome 4q."

An assay for determining whether a putative or actual agent can bind to NPY is presented in WO-A-98/52890 (see page 96 thereof, lines 2 to 28).

35 I: NPYs (in particular NPY antagonists) are discussed in the following review articles:

Dunlop J, Rosenzweig-Lipson S : Therapeutic approaches to obesity Exp Opin Ther Pat 1999 8 12 1683 -1694



Wang S, Ferguson KC, Burris TP, Dhurandhar NV: 8th annual international conference on obesity and non-insulin dependent diabetes mellitus: novel drug developments. Exp Opin Invest Drugs 1999 8 7 1117 -1125

5 Ling AL : Neuropeptide Y receptor antagonists Exp Opin Ther Pat 1999 9 4 375-384  
Adham N, Tamm J, Du P, Hou C, et al : Identification of residues involved in the binding of the antagonist SNAP 6608 to the Y5 receptor Soc Neurosci Abstr 1998 24 part 2 626.9

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Criscione L, Rigollier P, Batzl-Hartmann C, Rueger H, Stricker-Krongrad A, et al : Food intake in free-feeding and energy-deprived lean rats is mediated by the  
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Neurogen Corp : NGD 95-1 Clin Trials Monitor 1996 5 10 Ab 19244

Buttle LA : Anti-obesity drugs: on target for huge market sales. Exp Opin Invest  
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Gehlert DR, Hipskind PA : Neuropeptide Y receptor antagonists in obesity. Exp Opin Invest Drugs 1996 7 9 1827 -1838

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Zarrinmayeh H, Zimmerman DM, Cantrell BE, Schober DA, Bruns RF, Gackenhaimer SL, Ornstein PL, Hipkind PA, Britton TC, Gehlert DR : Structure-activity relationship of a series of diaminoalkyl substituted benzimidazole as neuropeptide Y Y1 receptor antagonists *Bioorganic Med Chem Lett* 1999 9 5 647 -652

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Rudolf K, Eberlein W, Engel W, Wieland HA, Willim KD, Entzeroth M, Wienen W, Beck Sickinger AG, Doods HN : The first highly potent and selective non-peptide neuropeptide YY1 receptor antagonist: BIBP3226 *Eur J Pharmacol* 1994 271 2-3 R11 -R13

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Wieland HA, Willim KD, Entzeroth M, Wienen W, Rudolf K, Eberlein W, Engel W, Doods HN : Subtype selectivity and antagonist profile of the nonpeptide neuropeptide Y1 receptor antagonist BIBP 3226 *J Pharmacol Exp Ther* 1995 275 1 143 –149.

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R11 -R13

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Hong Y, Gregor V, Ling AL, Tompkins EV, Porter J, Chou TS, Paderes G, Peng Z,  
Hagaman C, Anderes K, Luthin D, May J : Synthesis and biological evaluation of  
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Anaheim MEDI 108

I:NPYs (in particular NPY antagonists) are disclosed in the following documents:

WO-98/07420

WO-94/00486

WO-96/22305

WO-97/20821

WO-97/20822

WO-96/14307

JP-07267988

WO-96/12489

US-5552422

WO-98/35957

WO-96/14307

WO-94/17035

EP-0614911

WO-98/40356

EP-0448765

EP-0747356

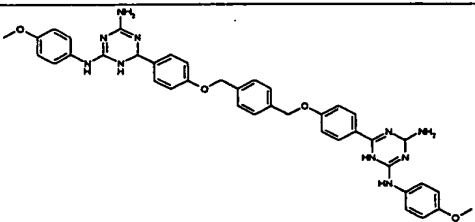
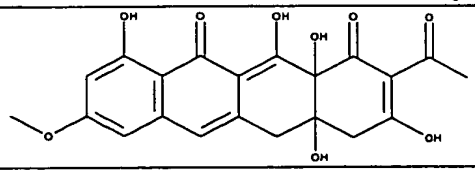
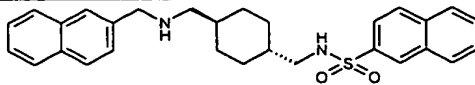
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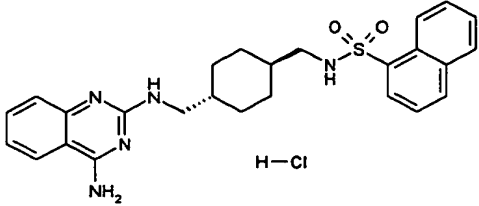
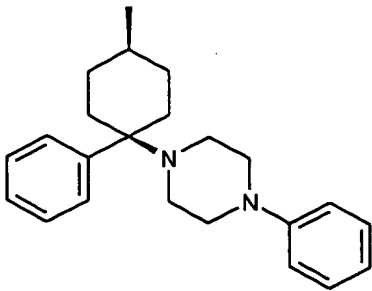
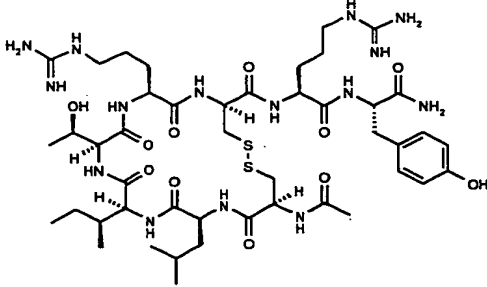
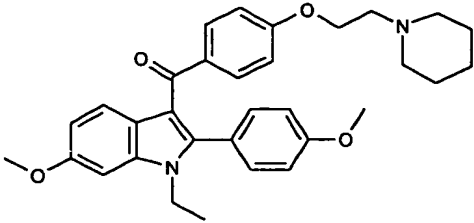
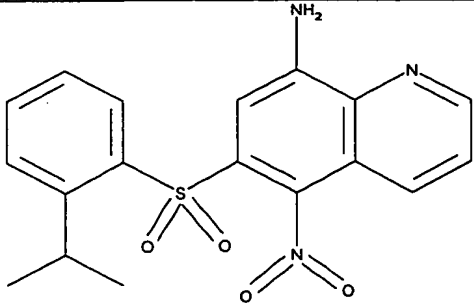
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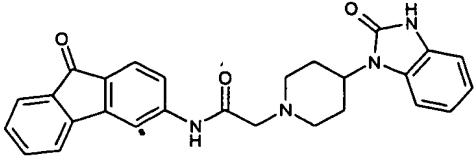
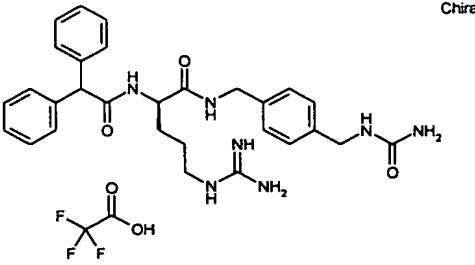
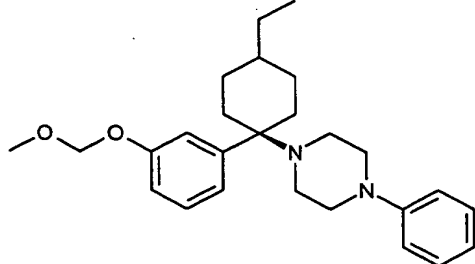
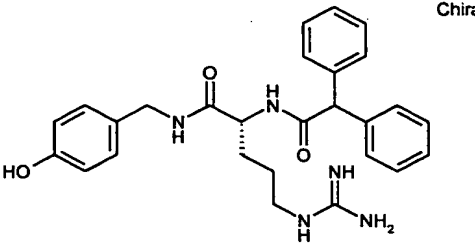
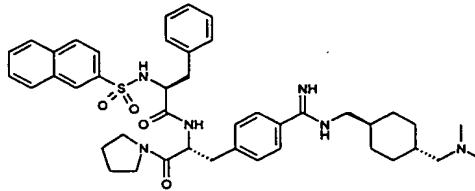
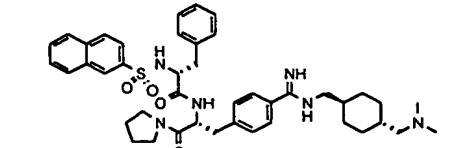
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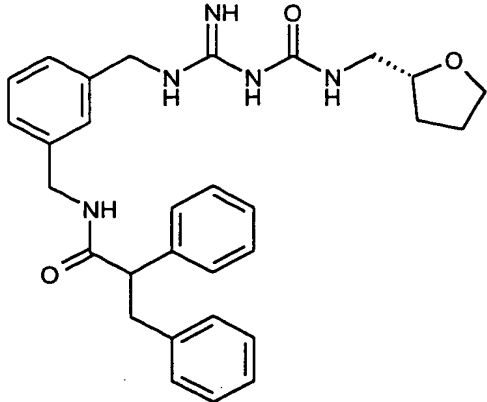
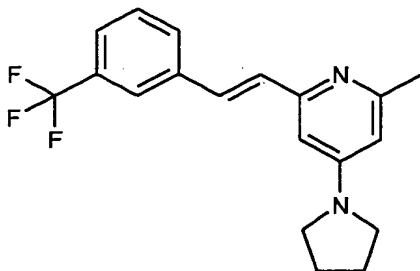
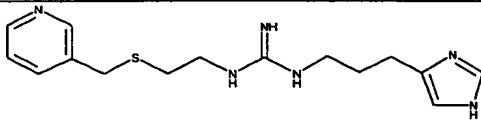
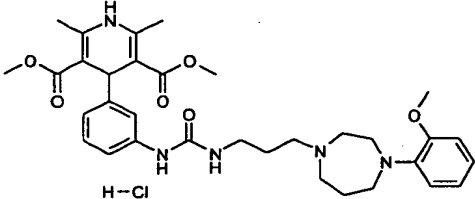
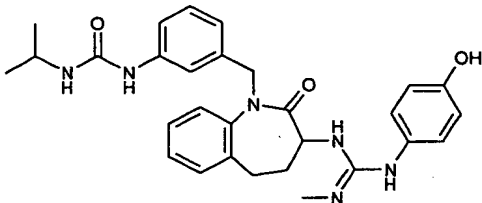
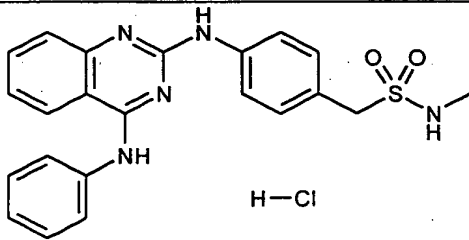
Preferred examples of I:NPYs are selected from the following structures.

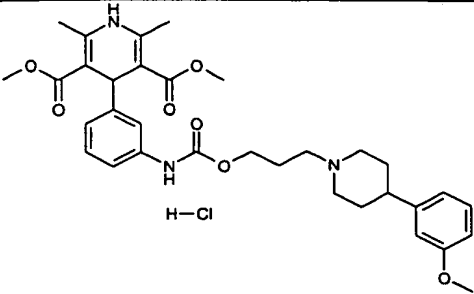
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<u>Compound</u>	<u>Structure</u>	<u>Mode of Action</u> <u>References</u>
F34		I:NPY Y1 WO-98/07420 Ref 3
F35		I:NPY Ref 5
F36		I:NPY Y5 Ref 1, 4

F37	<p>Ile - Cys- Pro-</p> <p>Cys- Tyr- Arg- Leu- Arg- Tyr-</p> <p>NH<sub>2</sub> cyclic (2,2'), (4,4')- disulfide dimer</p>	<p>I:NPY Y1</p> <p>WO-94/00486</p> <p>WO-96/22305</p> <p>Ref 1,2, 23</p>
F38	 <p>H-Cl</p>	<p>I:NPY Y5</p> <p>WO-97/20821</p> <p>WO-97/20822</p> <p>Ref 1, 3, 6, 7</p>
F39		<p>I:NPY Y1</p> <p>WO-96/14307</p> <p>Ref 1, 8, 9, 10, 11</p>
F40		<p>I:NPY Y1</p> <p>JP-07267988</p> <p>Ref 1</p>
F41		<p>I:NPY Y1</p> <p>WO-96/12489</p> <p>Ref 3, 12, 13, 14, 15, 16, 17</p>
F42		<p>I:NPY Y1</p> <p>US-5552422</p> <p>Ref 17, 18, 19, 20</p>

F43		I:NPY Y5 WO-98/35957 Ref 3
F44	 Chiral	I:NPY Y1 Ref 21, 22
F45		I:NPY Y1 WO-96/14307 Ref 3
F46	For formula, see reference	I:NPY Y1 Ref 24
F47a	 Chiral	I:NPY Y1 WO-94/17035 Ref 3, 17, 25, 26
F47b	For formula, see reference	I:NPY Y1 Ref 3, 12, 13, 14, 15, 16, 17
F48		I:NPY Y1 EP-0614911 Ref 27
F49		I:NPY Y1 EP-0614911 Ref 27

F50		I:NPY Y1 Ref 28
F51		I:NPY Y5 WO-98/40356
F52		I:NPY EP-0448765
F53		I:NPY Y1 EP-0747356
F54		I:NPY Y1 WO-98/35941
F55		I:NPY Y5 WO-97/46250

F56	 <chem>COC(=O)c1c(C)c2c(c1)nc(C)c2C(=O)OC(=O)Nc3ccc(cc3)COC(=O)OCCCN4CCc5ccc(OC)cc5CC4</chem> H-Cl	I:NPY Y1 EP-0747357
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ENDOGENOUS cAMP

5 In a highly preferred embodiment the agent of the present invention potentiates endogenous cAMP – such as potentiates endogenous cAMP levels.

Here, the term “endogenous cAMP” means cAMP that arises from sexual stimulation (sexual arousal). Hence, the term does not encompass cAMP levels that will be  
10 elevated independent of sexual drive.

Thus, according to the present invention, treatment of MED is achieved by directly or indirectly potentiating endogenous cAMP signalling which, in turn, increases male genital blood flow; thus enhancing the natural sexual arousal response. Thus, the  
15 treatment method of the present invention restores or potentiates the normal arousal response.

In the treatment method of the present invention, this result may be achieved by use of a NPY receptor antagonist.

20

An animal test model is provided herein. This animal test model may be used to determine increases of genital blood flow as a result of cAMP potentiation. In this animal model a pelvic nerve is stimulated – which brings on an effect that mimics the physiology of a sexual arousal/response. In these experiments, agents according to  
25 the present invention cause an increase in blood flow, above control increases, after the nerve has been stimulated. In the absence of stimulation, the agents have no (or a negligible) effect in causing an increase in blood flow. Typically, in these experiments, the nerve is stimulated in order to obtain a base line increase in blood flow. Then a candidate (or actual) agent is delivered to the animal systemically or  
30 locally, such as by the intravenous, topical or oral route. An increase in blood flow, compared to control increases, is then indicative of an agent according to the present invention.

35 The agent of the present invention may also be used in combination with one or more additional “auxiliary” pharmaceutically active agents. The additional “auxiliary” pharmaceutically active agent, if either present or used in conjunction with

the agent of the present invention, may be referred to as an "additional agent". One or more of these additional agents may be one or more of: I:PDE, an I:NEP, another I:NPY.

5 Further combinations of agents are discussed in more detail below.

If the "auxiliary" additional agent of the present invention is an I:PDE then for some embodiments said PDE is a cAMP hydrolysing PDE (and optionally cGMP hydrolysing). The term "hydrolysing cAMP" also includes metabolising and/or  
10 breaking down cAMP. The term "hydrolysing cAMP (and optionally cGMP)" means that the additional agent may be able to hydrolyse cGMP in addition to cAMP. Here, the term "hydrolyse cGMP" also includes metabolising and/or breaking down cGMP. However, for some embodiments of the present invention, it is to be understood that the additional agent need not necessarily be able to hydrolyse cGMP.

15

#### PDE5 Inhibitors

As detailed hereinbefore according to a further embodiment of the present invention there is provided use of a pharmaceutical composition comprising an NPYi and an  
20 I:PDE<sub>cGMP</sub> for use in the treatment of MED. More particularly the present invention provides use of a pharmaceutical composition comprising an NPYi and an I:PDE5<sub>cGMP</sub> in the manufacture of a medicament for the treatment of MED.

Suitable PDE5i's for use in the pharmaceutical compositions according to the present  
.5 invention are the cGMP PDE5i's hereinafter detailed. Particularly preferred for use herein are potent and selective cGMP PDE5i's.

Suitable cGMP PDE5 inhibitors for the use according to the present invention include:

30

the pyrazolo [4,3-d]pyrimidin-7-ones disclosed in EP-A-0463756; the pyrazolo [4,3-d]pyrimidin-7-ones disclosed in EP-A-0526004; the pyrazolo [4,3-d]pyrimidin-7-ones disclosed in published international patent application WO 93/06104; the isomeric pyrazolo [3,4-d]pyrimidin-4-ones disclosed in published international patent  
35 application WO 93/07149; the quinazolin-4-ones disclosed in published international patent application WO 93/12095; the pyrido [3,2-d]pyrimidin-4-ones disclosed in published international patent application WO 94/05661; the purin-6-ones disclosed

in published international patent application WO 94/00453; the pyrazolo [4,3-d]pyrimidin-7-ones disclosed in published international patent application WO 98/49166; the pyrazolo [4,3-d]pyrimidin-7-ones disclosed in published international patent application WO 99/54333; the pyrazolo [4,3-d]pyrimidin-4-ones disclosed in  
5 EP-A-0995751; the pyrazolo [4,3-d]pyrimidin-7-ones disclosed in published international patent application WO 00/24745; the pyrazolo [4,3-d]pyrimidin-4-ones disclosed in EP-A-0995750; the compounds disclosed in published international application WO95/19978; the compounds disclosed in published international application WO 99/24433 and the compounds disclosed in published international  
10 application WO 93/07124.

It is to be understood that the contents of the above published patent applications, and in particular the general formulae and exemplified compounds therein are incorporated herein in their entirety by reference thereto.

15 Preferred type V phosphodiesterase inhibitors for the use according to the present invention include:

5-[2-ethoxy-5-(4-methyl-1-piperazinylsulphonyl)phenyl]-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (sildenafil) also known as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulphonyl]-4-methylpiperazine (see EP-A-0463756);  
20

5-(2-ethoxy-5-morpholinoacetylphenyl)-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see EP-A-0526004);  
25

3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-n-propoxyphenyl]-2-(pyridin-2-yl)methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see WO98/49166);

3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-(2-methoxyethoxy)pyridin-3-yl]-2-(pyridin-2-yl)methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see WO99/54333);  
30

(+)-3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-(2-methoxy-1(R)-methylethoxy)pyridin-3-yl]-2-methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one, also known as 3-ethyl-5-[5-[4-ethylpiperazin-1-ylsulphonyl]-2-((1R)-2-methoxy-1-  
35

methylethyl]oxy)pyridin-3-yl]-2-methyl-2,6-dihydro-7H-pyrazolo[4,3-d] pyrimidin-7-one  
(see WO99/54333);

5- [2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-[2-methoxyethyl]-  
2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one, also known as 1-{6-ethoxy-5-[3-ethyl-  
6,7-dihydro-2-(2-methoxyethyl)-7-oxo-2H-pyrazolo[4,3-d]pyrimidin-5-yl]-3-  
pyridylsulphonyl}-4-ethylpiperazine (see PDE5 Example 1 hereinafter);

5-[2-*iso*-Butoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-(1-  
methylpiperidin-4-yl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see PDE5  
Example 2 hereinafter);

5-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-phenyl-2,6-  
dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see PDE5 Example 3 hereinafter);  
15

5-(5-Acetyl-2-propoxy-3-pyridinyl)-3-ethyl-2-(1-isopropyl-3-azetidiny)-2,6-dihydro-7H-  
pyrazolo[4,3-d]pyrimidin-7-one (see PDE5 Example 4 hereinafter);

5-(5-Acetyl-2-butoxy-3-pyridinyl)-3-ethyl-2-(1-ethyl-3-azetidiny)-2,6-dihydro-7H-  
pyrazolo[4,3-d]pyrimidin-7-one (see PDE5 Example 5 hereinafter);  
20

(6R,12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylenedioxyphenyl) -  
pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione (IC-351), i.e. the compound of  
examples 78 and 95 of published international application WO95/19978, as well as  
the compound of examples 1, 3, 7 and 8;  
25

2-[2-ethoxy-5-(4-ethyl-piperazin-1-yl-1-sulphonyl)-phenyl]-5-methyl-7-propyl-3H-  
imidazo[5,1-f][1,2,4]triazin-4-one (vardeafil) also known as 1-[[3-(3,4-dihydro-5-  
methyl-4-oxo-7-propylimidazo[5,1-f]-as-triazin-2-yl)-4-ethoxyphenyl]sulphonyl]-4-  
ethylpiperazine, i.e. the compound of examples 20, 19, 337 and 336 of published  
international application WO99/24433; and  
30

the compound of example 11 of published international application WO93/07124  
(EISAI); and  
35

compounds 3 and 14 from Rotella D P, *J. Med. Chem.*, 2000, 43, 1257.

Still other type cGMP PDE5 inhibitors useful in conjunction with the present invention include: 4-bromo-5-(pyridylmethylamino)-6-[3-(4-chlorophenyl)-propoxy]-3(2H)pyridazinone; 1-[4-[(1,3-benzodioxol-5-ylmethyl)amino]-6-chloro-2-quinazoliny]-4-piperidine-carboxylic acid, monosodium salt; (+)-cis-5,6a,7,9,9,9a-hexahydro-2-[4-(trifluoromethyl)-phenylmethyl-5-methyl-cyclopent-4,5]imidazo[2,1-b]purin-4(3H)one; furazlocillin; cis-2-hexyl-5-methyl-3,4,5,6a,7,8,9,9a-octahydrocyclopent[4,5]-imidazo[2,1-b]purin-4-one; 3-acetyl-1-(2-chlorobenzyl)-2-propylindole-6-carboxylate; 3-acetyl-1-(2-chlorobenzyl)-2-propylindole-6-carboxylate; 4-bromo-5-(3-pyridylmethylamino)-6-(3-(4-chlorophenyl)propoxy)-3-(2H)pyridazinone; 1-methyl-5(5-morpholinoacetyl-2-n-propoxyphenyl)-3-n-propyl-1,6-dihydro-7H-pyrazolo(4,3-d)pyrimidin-7-one; 1-[4-[(1,3-benzodioxol-5-ylmethyl)amino]-6-chloro-2-quinazoliny]-4-piperidinecarboxylic acid, monosodium salt; Pharmaprojects No. 4516 (Glaxo Wellcome); Pharmaprojects No. 5051 (Bayer); Pharmaprojects No. 5064 (Kyowa Hakko; see WO 96/26940); Pharmaprojects No. 5069 (Schering Plough); GF-196960 (Glaxo Wellcome); E-8010 and E-4010 (Eisai); Bay-38-3045 & 38-9456 (Bayer) and Sch-51866.

The suitability of any particular cGMP PDE5 inhibitor can be readily determined by evaluation of its potency and selectivity using literature methods followed by evaluation of its toxicity, absorption, metabolism, pharmacokinetics, etc in accordance with standard pharmaceutical practice.

Preferably, the cGMP PDE5 inhibitors have an  $IC_{50}$  at less than 100 nanomolar, more preferably, at less than 50 nanomolar, more preferably still at less than 10 nanomolar.

$IC_{50}$  values for the cGMP PDE5 inhibitors may be determined using established literature methodology, for example as described in EP0463756-B1 and EP0526004-A1 and as detailed in the Test Methods Section hereinafter.

Preferably the cGMP PDE5 inhibitors used in the pharmaceutical compositions according to the present invention are selective for the PDE5 enzyme. Preferably they are selective over PDE3, more preferably over PDE3 and PDE4. Preferably, the cGMP PDE5 inhibitors of the invention have a selectivity ratio greater than 100 more preferably greater than 300, over PDE3 and more preferably over PDE3 and PDE4.

Selectivity ratios may readily be determined by the skilled person. IC50 values for the PDE3 and PDE4 enzyme may be determined using established literature methodology, see S A Ballard *et al*, Journal of Urology, 1998, vol. 159, pages 2164-2171 and as detailed herein after.

5

Highly preferred for use in combination with NPYi in the pharmaceutical compositions herein are potent and selective PDE5 inhibitors.

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Especially preferred herein is the combination of one or more potent and selective cGMP PDE5 inhibitors with one or more selective inhibitors of the NPY Y1 receptor.

#### ADDITIONAL ACTIVE AGENTS

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For some applications the agent of the present invention may be administered in conjunction with another pharmaceutically active agent. Here the co-administration need not be done at the same time, let alone by the same route. An example of a co-administration composition could be a composition that comprises an agent according to the present invention and an additional agent, wherein the additional agent could have a direct potentiating effect on cAMP. Combination examples are discussed *infra*.

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25

For some applications, preferably the additional agent has an indirect potentiating effect on cAMP. Examples of such additional agents include I:NEP and/or I:NPY. Alternatively expressed, for some applications, preferably the additional agent does not have a direct potentiating effect on cAMP. It is to be understood that the additional agent may have an indirect potentiating effect on cAMP by acting on naturally found and naturally located directly acting agents – such as naturally found and located VIP.

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For some applications, preferably the additional agent has a direct potentiating effect on cAMP. Examples of such additional agents include I:PDE.

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For some applications, the additional agent is an inhibitor – i.e. it is capable of exhibiting an inhibitory function.

For some applications, the additional agent is an antagonist.

For some applications, preferably the additional agent is an I:PDE (sometimes written as PDEi)

For some applications, preferably the additional agent is a selective I:PDE.

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For some applications, preferably the additional agent is an I:PDE1 or I:PDE2 (sometimes written as I:PDE11 or PDE11i or PDE2i) or I:PDE3 or I:PDE4 or I:PDE7 or I:PDE8, more preferably the agent is an I:PDE2.

10 For some applications, preferably the additional agent is a selective I:PDE11.

For some applications, preferably the additional agent is a I:NEP (sometimes written as NEPi).

15 For some applications, preferably the additional agent is a selective I:NEP.

For some applications, preferably the additional agent is a I:NEP wherein said NEP is EC 3.4.24.11.

20 For some applications, preferably the additional agent is a selective I:NEP wherein said NEP is EC 3.4.24.11.

For some applications, the agent does not cause - or is administered in such a fashion so that it does not cause - a prolonged drop in blood pressure (e.g. over a  
25 period of about 5 minutes or more). In this embodiment, if the agent is to be delivered topically then that agent may have the ability to cause a drop in blood pressure (such as if it were to be delivered intravenously), provided that in the topical application minimal levels of the agent pass into the blood stream. For an oral agent, it is preferred that the agent does not cause a prolonged drop in blood  
30 pressure.

In a preferred aspect, the agent of the present invention does not cause - or is administered in such a fashion so that it does not cause - a large change in heart rate.

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## TREATMENT

It is to be appreciated that all references herein to treatment include one or more of curative, palliative and prophylactic treatment. Preferably, the term treatment includes at least curative treatment and/or palliative treatment.

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### SEXUAL STIMULATION

The present invention also encompasses use as defined hereinbefore via administration of a NPYi (and an PDE5i where applicable) before and/or during sexual stimulation. Here the term "sexual stimulation" may be synonymous with the term "sexual arousal". This aspect of the present invention is advantageous because it provides systemic selectivity. The natural cascade only occurs at the genitalia and not in other locations – e.g. in the heart etc. Hence, it would be possible to achieve a selective effect on the genitalia via the MED treatment according to the present invention.

15

Thus, according to the present invention it is highly desirable that there is a sexual stimulation step at some stage. We have found that this step can provide systemic selectivity. Here, "sexual stimulation" may be one or more of a visual stimulation, a physical stimulation, an auditory stimulation, or a thought stimulation.

20

Thus, preferably the agents of the present invention are delivered before or during sexual stimulation, particularly when those agents are for oral delivery.

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Hence, for this preferred aspect, the present invention provides for the use of an agent of the present invention in the manufacture of a medicament for the treatment of MED; wherein the agent is capable of potentiating cAMP in the sexual genitalia of a male suffering from MED; and wherein said male is sexually stimulated before or during administration of said medicament.

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Preferably, the present invention provides for the use of an agent of the present invention in the manufacture of a medicament for the treatment of MED; wherein the agent is capable of potentiating cAMP in the sexual genitalia of a male suffering from MED; wherein said male is sexually stimulated before or during administration of said medicament; and wherein said medicament is delivered orally to said male.

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In addition, for this preferred aspect, the present invention provides for a method of treating a male suffering from MED; the method comprising delivering to the male an agent of the present invention that is capable of potentiating cAMP in the sexual genitalia; wherein the agent is in an amount to cause potentiation of cAMP in the sexual genitalia of the male; wherein the agent is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient; and wherein said male is sexually stimulated before or during administration of said agent.

Preferably, the present invention provides for a method of treating a male suffering from MED; the method comprising delivering to the male an agent of the present invention that is capable of potentiating cAMP in the sexual genitalia; wherein the agent is in an amount to cause potentiation of cAMP in the sexual genitalia of the male; wherein the agent is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient; wherein said male is sexually stimulated before or during administration of said agent; and wherein said agent is delivered orally to said male.

#### POTENTIATING cAMP

As used herein with reference to cAMP, the term "potentiating" includes any one or more of: increasing the effectiveness of cAMP, increasing the levels of cAMP, increasing the activity of cAMP, decreasing the level of cAMP degradation, decreasing the level of cAMP inhibition.

The potentiating effect can be a direct effect. An example of a direct effect would be upregulation of cAMP levels by an agent that increases the expression thereof.

Alternatively, the potentiating effect could be an indirect effect. An example of such an effect would be action on a substance that would otherwise inhibit and/or reduce the levels and/or activity of cAMP. Another example of such an effect would be increasing the action of a substance that increases the effectiveness of cAMP, increases the levels of cAMP, increases the activity of cAMP, decreases the level of cAMP degradation, or decreases the level of cAMP inhibition.

An example of an additional PcAMP would be I:PDE, such as I:PDEII.

#### cAMP Mimetic

For some aspects of the present invention, the additional agent may act as a cAMP mimetic.

As used herein, the term "cAMP mimetic" means an agent that can act in a similar fashion (e.g. have a similar biological profile and effect) to cAMP in the male sexual genitalia and, in doing so, does any one or more of: increases the effectiveness of cAMP like moieties, increases the levels of cAMP like moieties, increases the activity of cAMP like moieties, decreases the level of degradation of cAMP like moieties, decreases the level of inhibition of cAMP like moieties.

An example of a cAMP mimetic would be forskolin. Here we have found that forskolin increases blood flow to the male genitalia and it can also act as a relaxant.

In a preferred aspect, the cAMP mimetic is administered orally.

#### ACTIVATOR OF cAMP

As used herein, the term "activator of cAMP" means a substance that controls or releases cAMP in the male sexual genitalia. The control may be direct (e.g. on cAMP itself) or indirect (e.g. via activation of cAMP). For ease of reference, we refer to these substances as  $A_{cAMP}$ .

#### TARGET

The term "target" as used herein with reference to the present invention means any substance that is cAMP, an  $A_{cAMP}$ , an  $I_{cAMP}$ , or an  $AM_{cAMP}$ . Otherwise expressed, the target of the present invention can be referred to as a  $P_{cAMP}$  target.

The target of the present invention and/or the additional target may be an amino acid sequence and/or a nucleotide sequence encoding same and/or an expression unit responsible for the expression of same and/or a modulator of same.

The target may even be a combination of such targets.

#### AGENT

Agents for use in the treatment of MED according to of the present invention may be any suitable agent that can act as a NPYi and, where appropriate an agent which can act as a PDE5i.

5 Such agents (i.e. the agents as defined above and/or the additional agents as defined hereinbefore) can be an amino acid sequence or a chemical derivative thereof. The substance may even be an organic compound or other chemical. The agent may even be a nucleotide sequence - which may be a sense sequence or an anti-sense sequence. The agent may even be an antibody.

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Thus, the term "agent" includes, but is not limited to, a compound which may be obtainable from or produced by any suitable source, whether natural or not.

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The agent may be designed or obtained from a library of compounds which may comprise peptides, as well as other compounds, such as small organic molecules, such as lead compounds.

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By way of example, the agent may be a natural substance, a biological macromolecule, or an extract made from biological materials such as bacteria, fungi, or animal (particularly mammalian) cells or tissues, an organic or an inorganic molecule, a synthetic agent, a semi-synthetic agent, a structural or functional mimetic, a peptide, a peptidomimetics, a derivatised agent, a peptide cleaved from a whole protein, or a peptides synthesised synthetically (such as, by way of example, either using a peptide synthesizer or by recombinant techniques or combinations thereof, a recombinant agent, an antibody, a natural or a non-natural agent, a fusion protein or equivalent thereof and mutants, derivatives or combinations thereof.

25

As used herein, the term "agent" may be a single entity or it may be a combination of agents.

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If the agent is an organic compound then for some applications - such as if the agent is a NEPi - that organic compound may typically comprise an amide group (i.e. -N(H)-C(O)- or even -C(O)-N(H)-) and one or more hydrocarbyl groups. Here, the term "hydrocarbyl group" means a group comprising at least C and H and may optionally comprise one or more other suitable substituents. Examples of such substituents may include halo-, alkoxy-, nitro-, an alkyl group, a cyclic group etc. In addition to the possibility of the substituents being a cyclic group, a combination of

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substituents may form a cyclic group. If the hydrocarbyl group comprises more than one C then those carbons need not necessarily be linked to each other. For example, at least two of the carbons may be linked *via* a suitable element or group. Thus, the hydrocarbyl group may contain hetero atoms. Suitable hetero atoms will be apparent to those skilled in the art and include, for instance, sulphur, nitrogen and oxygen. For some applications, preferably the agent comprises at least one cyclic group. For some applications, preferably the agent comprises at least one cyclic group linked to another hydrocarbyl group via an amide bond. Examples of such compounds are presented in the Additional Compound Examples section herein.

If the agent is an organic compound then for some applications - such as if the agent is an PDE5i – that organic compound may typically comprise two or more linked hydrocarbyl groups. For some applications, preferably the agent comprises at least two cyclic groups – wherein one of which cyclic groups may be a fused cyclic ring structure. For some applications, preferably at least one of the cyclic groups is a heterocyclic group. For some applications, preferably the heterocyclic group comprises at least one N in the ring. Examples of such compounds are presented in the PDE5 Examples section herein.

If the agent is an organic compound then for some applications - such as if the agent is an I:NPY – that organic compound may typically comprise two or more linked hydrocarbyl groups. For some applications, preferably the agent comprises at least two cyclic groups – optionally wherein one of which cyclic groups may be a fused cyclic ring structure. For some applications, at least one of the cyclic groups is a heterocyclic group. For some applications, preferably the heterocyclic group comprises at least one N in the ring. Examples of such compounds are presented in the Additional Examples section herein.

The agent may contain halo groups. Here, "halo" means fluoro, chloro, bromo or iodo.

The agent may contain one or more of alkyl, alkoxy, alkenyl, alkylene and alkenylene groups – which may be unbranched- or branched-chain.

The agent may be in the form of a pharmaceutically acceptable salt – such as an acid addition salt or a base salt – or a solvate thereof, including a hydrate thereof. For a review on suitable salts see Berge *et al*, J. Pharm. Sci., 1977, 66, 1-19.

Suitable acid addition salts are formed from acids which form non-toxic salts and examples are the hydrochloride, hydrobromide, hydroiodide, sulphate, bisulphate, nitrate, phosphate, hydrogen phosphate, acetate, maleate, fumarate, lactate, tartrate, citrate, gluconate, succinate, saccharate, benzoate, methanesulphonate, ethanesulphonate, benzenesulphonate, *p*-toluenesulphonate and pamoate salts.

Suitable base salts are formed from bases which form non-toxic salts and examples are the sodium, potassium, aluminium, calcium, magnesium, zinc and diethanolamine salts.

A pharmaceutically acceptable salt of an agent as defined hereinbefore may be readily prepared by mixing together solutions of the agent and the desired acid or base, as appropriate. The salt may precipitate from solution and be collected by filtration or may be recovered by evaporation of the solvent.

The agent may exist in polymorphic form.

The agent may contain one or more asymmetric carbon atoms and therefore exists in two or more stereoisomeric forms. Where an agent contains an alkenyl or alkenylene group, *cis* (E) and *trans* (Z) isomerism may also occur. The present invention includes the individual stereoisomers of the agent and, where appropriate, the individual tautomeric forms thereof, together with mixtures thereof.

Separation of diastereoisomers or *cis* and *trans* isomers may be achieved by conventional techniques, e.g. by fractional crystallisation, chromatography or H.P.L.C. of a stereoisomeric mixture of the agent or a suitable salt or derivative thereof. An individual enantiomer of the agent may also be prepared from a corresponding optically pure intermediate or by resolution, such as by H.P.L.C. of the corresponding racemate using a suitable chiral support or by fractional crystallisation of the diastereoisomeric salts formed by reaction of the corresponding racemate with a suitable optically active acid or base, as appropriate.

The present invention also includes all suitable isotopic variations of the agent or a pharmaceutically acceptable salt thereof. An isotopic variation of an agent of the present invention or a pharmaceutically acceptable salt thereof is defined as one in which at least one atom is replaced by an atom having the same atomic number but

an atomic mass different from the atomic mass usually found in nature. Examples of isotopes that can be incorporated into the agent and pharmaceutically acceptable salts thereof include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorus, sulphur, fluorine and chlorine such as  $^2\text{H}$ ,  $^3\text{H}$ ,  $^{13}\text{C}$ ,  $^{14}\text{C}$ ,  $^{15}\text{N}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ,  $^{31}\text{P}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^{18}\text{F}$  and  $^{36}\text{Cl}$ , respectively. Certain isotopic variations of the agent and pharmaceutically acceptable salts thereof, for example, those in which a radioactive isotope such as  $^3\text{H}$  or  $^{14}\text{C}$  is incorporated, are useful in drug and/or substrate tissue distribution studies. Tritiated, i.e.,  $^3\text{H}$ , and carbon-14, i.e.,  $^{14}\text{C}$ , isotopes are particularly preferred for their ease of preparation and detectability. Further, substitution with isotopes such as deuterium, i.e.,  $^2\text{H}$ , may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased *in vivo* half-life or reduced dosage requirements and hence may be preferred in some circumstances. Isotopic variations of the agent and pharmaceutically acceptable salts thereof can generally be prepared by conventional procedures using appropriate isotopic variations of suitable reagents.

It will be appreciated by those skilled in the art that the agent may be derived from a prodrug. Examples of prodrugs include entities that have certain protected group(s) and which may not possess pharmacological activity as such, but may, in certain instances, be administered (such as orally or parenterally) and thereafter metabolised in the body to form the agent which are pharmacologically active.

It will be further appreciated that certain moieties known as "pro-moieties", for example as described in "Design of Prodrugs" by H. Bundgaard, Elsevier, 1985 (the disclosed of which is hereby incorporated by reference), may be placed on appropriate functionalities of the agents. Such prodrugs are also included within the scope of the invention.

The term inhibitor as used herein in relation to the NPYi and PDE5i compounds is to be regarded as being interchangeable with the term antagonist. Further the phrase, enhancing the endogenous erectile process, is to be regarded as being interchangeable with the phrase upregulation of the endogenous erectile process.

The  $P_{\text{cAMP}}$  may do any one or more of: directly or indirectly increase the effectiveness of cAMP, directly or indirectly increase the levels of cAMP, directly or indirectly increase the activity of cAMP, directly or indirectly decrease the level of cAMP degradation, directly or indirectly decrease the level of cAMP inhibition.

Preferably, the agent of the present invention directly or indirectly increases cAMP levels in the sexual genitalia of a male suffering from MED.

- 5 More preferably, the agent of the present invention directly or indirectly selectively increases cAMP levels in the sexual genitalia of a male suffering from MED.

More preferably, the agent of the present invention directly or indirectly selectively increases cAMP levels wherein said cAMP is sexually arousal induced cAMP.

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In a highly preferred aspect, the agent of the present invention of the present invention increases the relative amount of sexual arousal induced cAMP.

For some applications, the agent of the present invention selectively treats MED.

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In one aspect, the agent may inhibit or antagonise a suitable target and in doing so potentiate cAMP levels in the male sexual genitalia. In the text, we have used the term inhibitor to mean an inhibitor and/or antagonist.

- 20 In another aspect, the agent may activate or agonise a suitable target and in doing so potentiate cAMP levels in the male sexual genitalia. In the text, we have used the terms activator and upregulator inhibitor to mean activator and/or upregulator and/or agonist.

- 25 Thus, the agent may agonise, antagonise, upregulate, or inhibit a suitable target.

The agent of the present invention may be a single entity that is capable of exhibiting two or more of these properties. Alternatively, or in addition, the agent of the present invention can be a combination of agents that are capable of exhibiting one or more  
30 of these properties.

Preferably, the agent may selectively agonise, selectively antagonise, selectively upregulate, or selectively inhibit a suitable target.

- 35 Preferably, the agent may selectively agonise, selectively antagonise, selectively upregulate, or selectively inhibit a selective, suitable target.

The agent may also be capable of displaying one or more other beneficial functional properties. By way of example, the agent of the present invention may potentiate cAMP as well as potentiating cGMP.

5 For some applications (such as a topical application), the agent may also display an ACE (angiotensin converting enzyme) inhibitory action. An ACE assay is presented in the Experimental Section herein. For some applications (such as with particular patient types), such agents (i.e. those that also display ACE inhibitory action) may not be suitable for oral administration.

10 For some applications, the agent may also display an ECE (endothelium converting enzyme) inhibitory action. ECE assays are well known in the art.

#### PHARMACEUTICAL COMBINATIONS

15 As discussed hereinbefore treatment of MED according to the present invention may be achieved via a combination of a NPYi, and a PDE5i where present, with one or more other additional "auxiliary" pharmaceutically active agents, such as a nitric oxide donor, or a nitric oxide precursor eg L-arginine or inhibitors of arginase) and/or  
20 a centrally acting pharmaceutical (e.g. a dopamine receptor agonist such as apomorphine or selective dopamine D2 receptor agonists such as PNU-95666 or a melanocortin receptor agonist, such as melanotan II). Teachings on the use of apomorphine as a pharmaceutical may be found in US-A-5945117. In that particular document, apomorphine is delivered sub-lingually. In addition, or in the alternative,  
25 the agent may be used in combination with one or more of: one or more of a nitric oxide donor (eg NMI-921), one or more of a dopamine receptor agonist (eg apomorphine, Uprima, Ixsene), one or more of a heterocyclic amine such as generically and specifically disclosed in WO 00/40226, in particular example numbers 7, 8 and 9, one or more of a melanocortin receptor agonist (eg Melanotan II or PT14),  
30 one or more of a potassium channel opener (eg a  $K_{ATP}$  channel opener (eg minoxidil, nicorandil) and/or a calcium activated potassium channel opener (eg BMS-204352), one or more of an  $\alpha$ 1-adrenoceptor antagonist (eg phentolamine, Vasomax), one or more of a VIP receptor agonist or a VIP analogue (eg Ro-125-1553) or a VIP fragment, one or more of a  $\alpha$ -adrenoceptor antagonist with VIP combination (eg  
35 Invicorp, Aviptadil), one or more of a  $\alpha$ 2-adrenoceptor antagonist (eg.yohimbine), one or more of a testosterone replacement agent (inc DHEA (dehydroandrostendione),



testosterone (Tostrelle) or a testosterone implant (Organon)), one or more of a testosterone/oestradiol agent one or more of an estrogen agonists eg such as the compounds described in WO 96/21656, and preferably thereof Lasofoxifene, one or more of a serotonin receptor agonist or antagonist (eg 5HT1A, 5HT2C, 5HT2A and 5HT3 receptor agonists and antagonists; as described in WO2000/28993), one or more of a prostanoid receptor agonist (eg Muse, alprostadiol, misoprostol), one or more of a purinergic receptor agonist (especially P2Y2 and P2Y4) one or more antidepressant agents (eg bupropion (Wellbutrin), mirtazapine, nefazodone).

If a combination of additional active agents are administered, then they may be administered simultaneously, separately or sequentially with or to the NPYi as detailed hereinbefore.

#### AUXILIARY AGENTS

For some applications, preferably the auxiliary agent for use herein is an NEPi wherein said NEP is EC 3.4.24.11.

For some applications, preferably the auxiliary agent for use herein is a selective NEPi wherein said NEP is EC 3.4.24.11.

Preferably the auxiliary agent for use in the treatment of MED according to the present invention is an inhibitor – i.e. it is capable of exhibiting an inhibitory function.

Preferably the auxiliary agent for use in the treatment of MED according to the present invention is capable of directly enhancing the endogenous erectile process as detailed hereinbefore.

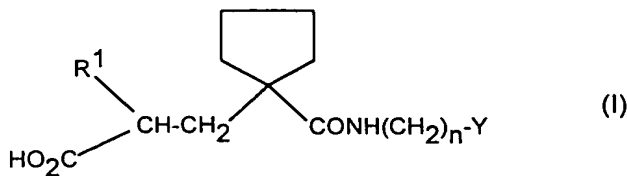
NEP EC3.4.24.11, also known as enkephalinase or neprilysin, is a zinc-dependent neutral endopeptidase. This enzyme is involved in the breakdown of several bioactive oligopeptides, cleaving peptide bonds on the amino side of hydrophobic amino acid residues (Reviewed in Turner et al., 1997). The key neuronally released bioactive agents or neuropeptides metabolised by NEP include natriuretic peptides such as atrial natriuretic peptides (ANP) as well as brain natriuretic peptide and C-type natriuretic peptide, bombesin, bradykinin, calcitonin gene-related peptide, endothelins, enkephalins, neurotensin, substance P and vasoactive intestinal

peptide. Some of these peptides have potent vasodilatory and neurohormone functions, diuretic and natriuretic activity or mediate behaviour effects.

Background teachings on NEP have been presented by Victor A. McKusick et al on <http://www3.ncbi.nlm.nih.gov/Omim/searchomim.htm>. The following information concerning NEP has been extracted from that source.

"Common acute lymphocytic leukemia antigen is an important cell surface marker in the diagnosis of human acute lymphocytic leukemia (ALL). It is present on leukemic cells of pre-B phenotype, which represent 85% of cases of ALL. CALLA is not restricted to leukemic cells, however, and is found on a variety of normal tissues. CALLA is a glycoprotein that is particularly abundant in kidney, where it is present on the brush border of proximal tubules and on glomerular epithelium. Letarte et al. (1988) cloned a cDNA coding for CALLA and showed that the amino acid sequence deduced from the cDNA sequence is identical to that of human membrane-associated neutral endopeptidase (NEP; EC 3.4.24.11), also known as enkephalinase. NEP cleaves peptides at the amino side of hydrophobic residues and inactivates several peptide hormones including glucagon, enkephalins, substance P, neurotensin, oxytocin, and bradykinin. By cDNA transfection analysis, Shipp et al. (1989) confirmed that CALLA is a functional neutral endopeptidase of the type that has previously been called enkephalinase. Barker et al. (1989) demonstrated that the CALLA gene, which encodes a 100-kD type II transmembrane glycoprotein, exists in a single copy of greater than 45 kb which is not rearranged in malignancies expressing cell surface CALLA. The gene was located to human chromosome 3 by study of somatic cell hybrids and in situ hybridization regionalized the location to 3q21-q27. Tran-Paterson et al. (1989) also assigned the gene to chromosome 3 by Southern blot analysis of DNA from human-rodent somatic cell hybrids. D'Adamio et al. (1989) demonstrated that the CALLA gene spans more than 80 kb and is composed of 24 exons."

Preferred for NEP's for use as auxiliary agents in the treatment of MED according to the present invention are compounds of general formula I:



wherein

$R^1$  is  $C_{1-6}$ alkyl which may be substituted by one or more substituents, which may be the same or different, selected from the list: halo, hydroxy,  $C_{1-6}$  alkoxy,  $C_{2-6}$  hydroxyalkoxy,  $C_{1-6}$  alkoxy( $C_{1-6}$ alkoxy),  $C_{3-7}$ cycloalkyl,  $C_{3-7}$ cycloalkenyl, aryl, aryloxy, ( $C_{1-4}$ alkoxy)aryloxy, heterocyclyl, heterocyclyloxy,  $-NR^2R^3$ ,  $-NR^4COR^5$ ,  $-NR^4SO_2R^5$ ,  $-CONR^2R^3$ ,  $-S(O)_pR^6$ ,  $-COR^7$  and  $-CO_2(C_{1-4}$ alkyl); or  $R^1$  is  $C_{3-7}$ cycloalkyl, aryl or heterocyclyl, each of which may be substituted by one or more substituents from said list, which substituents may be the same or different, which list further includes  $C_{1-6}$ alkyl; or  $R^1$  is  $C_{1-6}$  alkoxy,  $-NR^2R^3$  or  $-NR^4SO_2R^5$ ;

wherein

$R^2$  and  $R^3$  are each independently H,  $C_{1-4}$ alkyl,  $C_{3-7}$ cycloalkyl (optionally substituted by hydroxy or  $C_{1-4}$ alkoxy), aryl, ( $C_{1-4}$ alkyl)aryl,  $C_{1-6}$ alkoxyaryl or heterocyclyl; or  $R^2$  and  $R^3$  together with the nitrogen to which they are attached form a pyrrolidinyl, piperidino, morpholino, piperazinyl or  $N$ -( $C_{1-4}$ alkyl)piperazinyl group;

$R^4$  is H or  $C_{1-4}$ alkyl;

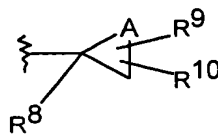
$R^5$  is  $C_{1-4}$ alkyl,  $CF_3$ , aryl, ( $C_{1-4}$ alkyl)aryl, ( $C_{1-4}$ alkoxy)aryl, heterocyclyl,  $C_{1-4}$ alkoxy or  $-NR^2R^3$  wherein  $R^2$  and  $R^3$  are as previously defined;

$R^6$  is  $C_{1-4}$ alkyl, aryl, heterocyclyl or  $NR^2R^3$  wherein  $R^2$  and  $R^3$  are as previously defined; and

$R^7$  is  $C_{1-4}$ alkyl,  $C_{3-7}$ cycloalkyl, aryl or heterocyclyl;  $n$  is 0, 1 or 2;  $p$  is 0, 1, 2 or 3;

the  $-(CH_2)_n-$  linkage is optionally substituted by  $C_{1-4}$ alkyl,  $C_{1-4}$ alkyl substituted with one or more fluoro groups or phenyl,  $C_{1-4}$ alkoxy, hydroxy, hydroxy( $C_{1-3}$ alkyl),  $C_{3-7}$ cycloalkyl, aryl or heterocyclyl;

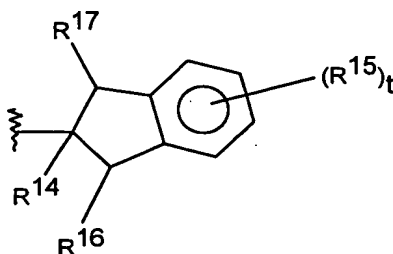
$Y$  is the group



wherein A is  $-(CH_2)_q-$  where q is 1, 2, 3 or 4 to complete a 3 to 7 membered carbocyclic ring which may be saturated or unsaturated;  $R^8$  is H,  $C_{1-6}$ alkyl,  $-CH_2OH$ , phenyl, phenyl( $C_{1-4}$ alkyl) or  $CONR^{11}R^{12}$ ;  $R^9$  and  $R^{10}$  are each independently H,  $-CH_2OH$ ,  $-C(O)NR^{11}R^{12}$ ,  $C_{1-6}$ alkyl, phenyl optionally substituted by  $C_{1-4}$ alkyl, or phenyl( $C_{1-4}$ alkyl) wherein the phenyl group is optionally substituted by  $C_{1-4}$ alkyl, or  $R^9$  and  $R^{10}$  together form a dioxolane;  $R^{11}$  and  $R^{12}$  which may be the same or different are H,  $C_{1-4}$ alkyl,  $R^{13}$  or  $S(O)_rR^{13}$ , where r is 0, 1 or 2 and  $R^{13}$  is phenyl optionally substituted by  $C_{1-4}$ alkyl or phenyl( $C_{1-4}$ alkyl) wherein the phenyl is optionally substituted by  $C_{1-4}$ alkyl; or

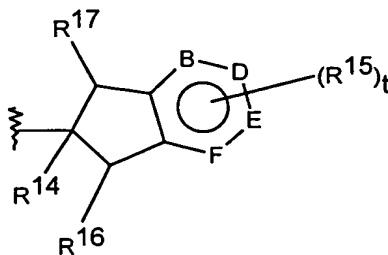
Y is the group,  $-C(O)NR^{11}R^{12}$  wherein  $R^{11}$  and  $R^{12}$  are as previously defined except that  $R^{11}$  and  $R^{12}$  are not both H; or

Y is the group,



wherein  $R^{14}$  is H,  $CH_2OH$ , or  $C(O)NR^{11}R^{12}$  wherein  $R^{11}$  and  $R^{12}$  are as previously defined; when present  $R^{15}$ , which may be the same or different to any other  $R^{15}$ , is OH,  $C_{1-4}$ alkyl,  $C_{1-4}$ alkoxy, halo or  $CF_3$ ; t is 0, 1, 2, 3 or 4; and  $R^{16}$  and  $R^{17}$  are independently H or  $C_{1-4}$ alkyl; or

Y is the group



wherein one or two of B, D, E or F is a nitrogen, the others being carbon; and  $R^{14}$  to  $R^{17}$  and t are as previously defined; or

Y is an optionally substituted 5-7 membered heterocyclic ring, which may be saturated, unsaturated or aromatic and contains a nitrogen, oxygen or sulphur and optionally one, two or three further nitrogen atoms in the ring and which may be optionally benzofused and optionally substituted by:

C<sub>1-6</sub> alkoxy; hydroxy; oxo; amino; mono or di-(C<sub>1-4</sub>alkyl)amino; C<sub>1-4</sub>alkanoylamino; or

C<sub>1-6</sub>alkyl which may be substituted by one or more substituents, which may be the same or different, selected from the list: C<sub>1-6</sub>alkoxy, C<sub>1-6</sub>haloalkoxy, C<sub>1-6</sub>alkylthio, halogen, C<sub>3-7</sub>cycloalkyl, heterocyclyl or phenyl; or

C<sub>3-7</sub>cycloalkyl, aryl or heterocyclyl, each of which may be substituted by one or more substituents, which may be the same or different, selected from the list: C<sub>1-6</sub>alkyl, C<sub>1-6</sub>alkoxy, C<sub>1-6</sub>haloalkoxy, C<sub>1-6</sub>alkylthio, halogen, C<sub>3-7</sub>cycloalkyl, heterocyclyl or phenyl;

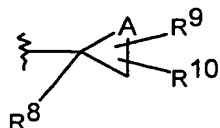
wherein when there is an oxo substitution on the heterocyclic ring, the ring only contains one or two nitrogen atoms and the oxo substitution is adjacent a nitrogen atom in the ring; or

Y is -NR<sup>18</sup>S(O)<sub>u</sub>R<sup>19</sup>, wherein R<sup>18</sup> is H or C<sub>1-4</sub>alkyl; R<sup>19</sup> is aryl, arylC<sub>1-4</sub>alkyl or heterocyclyl (preferably pyridyl); and

u is 0, 1, 2 or 3.

Preferably R<sup>1</sup> is C<sub>1-6</sub>alkyl, C<sub>1-6</sub>alkoxy, C<sub>1-6</sub>alkoxy(C<sub>1-3</sub>)alkyl, C<sub>1-6</sub>alkoxyC<sub>1-6</sub>alkoxyC<sub>1-3</sub>alkyl or C<sub>1-6</sub>alkyl substituted with aryl. Particularly preferred R<sup>1</sup> substituents are C<sub>1-6</sub>alkyl, C<sub>1-6</sub>alkoxy, C<sub>1-6</sub>alkoxy(C<sub>1-3</sub>)alkyl (especially methoxyethyl) or C<sub>1-6</sub>alkoxyC<sub>1-6</sub>alkoxyC<sub>1-3</sub>alkyl (especially methoxyethoxymethyl). It is especially preferred that R<sup>1</sup> is C<sub>1-4</sub>alkyl (preferably propyl).

When Y is the group



and the carbocyclic ring is fully saturated, then preferably one of  $R^9$  or  $R^{10}$  is  $-\text{CH}_2\text{OH}$ ,  $-\text{C}(\text{O})\text{NR}^{11}\text{R}^{12}$ ,  $\text{C}_{1-6}\text{alkyl}$ , phenyl optionally substituted by  $\text{C}_{1-4}\text{alkyl}$  or phenyl( $\text{C}_{1-4}\text{alkyl}$ ) wherein the phenyl group is optionally substituted by  $\text{C}_{1-4}\text{alkyl}$ .

More, preferably the carbocyclic ring is 5, 6 or 7 membered wherein one of  $R^9$  or  $R^{10}$ ,  $-\text{C}(\text{O})\text{NR}^{11}\text{R}^{12}$ , with the other being  $\text{C}_{1-6}\text{alkyl}$ , phenyl optionally substituted by  $\text{C}_{1-4}\text{alkyl}$  or phenyl( $\text{C}_{1-4}\text{alkyl}$ ) wherein the phenyl group is optionally substituted by  $\text{C}_{1-4}\text{alkyl}$ . More preferably,  $R^9$  and  $R^{10}$  are attached to adjacent carbon atoms in the ring. More preferably,  $R^8$  is  $\text{CH}_2\text{OH}$ .

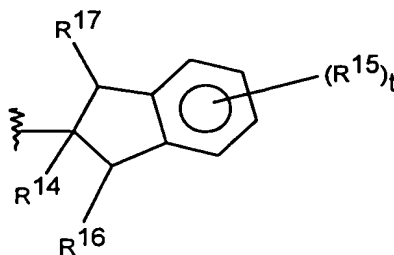
- 10 When Y is the group  $-\text{NR}^{18}\text{S}(\text{O})_u\text{R}^{19}$ , preferably  $R^{18}$  is H. More preferably,  $R^{19}$  is benzyl or phenyl. More preferably u is 2.

Preferably Y is the optionally substituted 5-7 membered heterocyclic ring. More preferably the ring is an optionally substituted aromatic ring, particularly pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, pyrazolyl, triazolyl, tetrazolyl, oxadiazolyl, thiazolyl, thiaiazolyl, oxazolyl, isoxazolyl, indolyl, isoindolyl, quinolyl, isoquinolyl, pyridonyl, quinoxaliny or quinazolinyl [especially oxadiazole (preferably 1,2,5- or 1,3,4-oxadiazole), pyridone (preferably 2-pyridone) or thiadiazole (preferably 1,3,4-thiadiazole) each of which may be substituted as defined in the first aspect.

- 20 Preferably the heterocyclic ring is substituted by one or more  $\text{C}_{1-6}\text{alkyl}$ , phenyl or phenyl $\text{C}_{1-4}\text{alkyl}$ , more preferably by  $\text{C}_{1-4}\text{alkyl}$  or benzyl. Preferably Y is an *N*-substituted pyridone, preferably by benzyl or  $\text{C}_{1-4}\text{alkyl}$ .

Preferably Y is a lactam linked at the nitrogen.

25 Preferably Y is



wherein  $R^{14}$  is preferably  $CH_2OH$  or  $C(O)NR^{11}R^{12}$ , especially  $C(O)NR^{11}R^{12}$ . Preferably  $R^{16}$  and  $R^{17}$  are hydrogen. Preferably  $t$  is 0.

The chiral carbon attached to  $R^1$  is preferably the R-enantiomer.

5

Particularly preferred NEPi compounds for as auxiliary agents for use in the treatment of MED according to the present invention (referred to hereinafter as the list of 10 preferred NEPi compounds) are as described in co-pending British Patent Application GB-A- 0106167.0 (appendix 1):

- 10 2-[(1-[(1-benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl)cyclopentyl)methyl]-4-methoxybutanoic acid (NEPi Example 35),  
 2-[(1-[(3-(2-oxo-1-pyrrolidinyl)propyl)amino]carbonyl)cyclopentyl]-methyl]-4-phenylbutanoic acid (NEPi Example 40),  
 (+)-2-[(1-[(2-(hydroxymethyl)-2,3-dihydro-1*H*-inden-2-yl)amino]carbonyl)cyclopentyl]-  
 15 methyl]-4-phenylbutanoic acid (NEPi Example 44),  
 2-[(1-[(5-methyl-1,3,4-thiadiazol-2-yl)amino]carbonyl)cyclopentyl)methyl]-4-phenylbutanoic acid (NEPi Example 43),  
 cis-3-(2-methoxyethoxy)-2-[(1-[(4-[(phenylsulfonyl)amino]carbonyl)cyclohexyl]-amino]carbonyl)cyclopentyl)methyl]propanoic acid (NEPi Example 38),  
 20 (+)-2-[(1-[(2-(hydroxymethyl)-2,3-dihydro-1*H*-inden-2-yl)amino]carbonyl)cyclopentyl]-methyl]pentanoic acid (NEPi Example 31),  
 (+)-2-[(1-[(5-ethyl-1,3,4-thiadiazol-2-yl)amino]carbonyl)cyclopentyl)methyl]pentanoic acid (NEPi Example 30),  
 2-[(1-[(3-benzylanilino)carbonyl)cyclopentyl)methyl]pentanoic acid (Example 21),  
 25 2-[(1-[(1-benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl)cyclopentyl)methyl]-pentanoic acid (NEPi Example 22), and  
 2-[(1-[(1*R*,3*S*,4*R*)-4-(aminocarbonyl)-3-butylcyclohexyl)amino]carbonyl)-cyclopentyl]methyl]pentanoic acid (NEPi Example 9).

- 30 In the above definition, unless otherwise indicated, alkyl groups having three or more carbon atoms may be straight or branched-chain. The term aryl as used herein means an aromatic hydrocarbon group such as phenyl or naphthyl which may optionally be substituted with, for example, one or more of OH, CN,  $CF_3$ ,  $C_1$ - $C_4$  alkyl,  $C_1$ - $C_4$  alkoxy, halo, carbamoyl, aminosulphonyl, amino, mono or di( $C_1$ - $C_4$  alkyl)amino or ( $C_1$ - $C_4$  alkanoyl)amino groups. Halo means fluoro, chloro, bromo or  
 35 iodo.

In the above definition, unless otherwise indicated the term heterocyclyl means a 5 or 6 membered nitrogen, oxygen or sulphur containing heterocyclic group which, unless otherwise stated, may be saturated, unsaturated or aromatic and which may optionally include a further oxygen or one to three nitrogen atoms in the ring and which may optionally be benzofused or substituted with for example, one or more halo, C<sub>1</sub>-C<sub>4</sub> alkyl, hydroxy, carbamoyl, benzyl, oxo, amino or mono or di-(C<sub>1</sub>-C<sub>4</sub> alkyl)amino or (C<sub>1</sub>-C<sub>4</sub> alkanoyl)amino groups. Particular examples of heterocycles include pyridyl, pyridonyl, pyrazinyl, pyrimidinyl, pyridazinyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, tetrazolyl, furanyl, tetrahydrofuranyl, tetrahydropyranyl, dioxanyl, thienyl, oxazolyl, isoxazolyl, thiazolyl, oxadiazolyl, thiadiazolyl, indolyl, isoindolyl, quinolyl, isoquinolyl, quinoxalyl, quinazolinyl and benzimidazolyl, each being optionally substituted as previously defined.

Details on a suitable assay system for identifying and/or studying an I:NEP are presented in the hereinafter in the section entitled NEP Assay.

Further examples of NEP inhibitors are disclosed and discussed in the following review articles:

- Pathol. Biol., 46(3), 1998, 191.  
Current Pharm. Design, 2(5), 1996, 443.  
Biochem. Soc. Trans., 21(3), 1993, 678.  
Handbook Exp. Pharmacol., 104/1, 1993, 547.  
TiPS, 11, 1990, 245.  
Pharmacol. Rev., 45(1), 1993, 87.  
Curr. Opin. Inves. Drugs, 2(11), 1993, 1175.  
Antihypertens. Drugs, (1997), 113.  
Chemtracts, (1997), 10(11), 804.  
Zinc Metalloproteases Health Dis. (1996), 105.  
Cardiovasc. Drug Rev., (1996), 14(2), 166.  
Gen. Pharmacol., (1996), 27(4), 581.  
Cardiovasc. Drug Rev., (1994), 12(4), 271.  
Clin. Exp. Pharmacol. Physiol., (1995), 22(1), 63.  
Cardiovasc. Drug Rev., (1991), 9(3), 285.  
Exp. Opin. Ther. Patents (1996), 6(11), 1147.



Yet, further examples of NEPi's are disclosed in the following documents:

EP-509442A

US-192435

US-4929641

EP-599444B

US-884664

EP-544620A

US-798684

J. Med. Chem. 1993, 3821.

Circulation 1993, 88(4), 1.

EP-136883

JP-85136554

US-4722810

Curr. Pharm. Design, 1996, 2, 443.

EP-640594

J. Med. Chem. 1993, 36(1), 87.

EP-738711-A

JP-270957

CAS # 115406-23-0

DE-19510566

DE-19638020

EP-830863

JP-98101565

EP-733642

WO9614293

JP-08245609

JP-96245609

WO9415908

JP05092948

WO-9309101

WO-9109840

EP-519738

EP-690070

J. Med. Chem. (1993), 36, 2420.

JP-95157459

Bioorg. Med. Chem. Letts., 1996, 6(1), 65.

Further I:NEPs are disclosed in the following documents:

EP-A-0274234

JP-88165353

Biochem.Biophys.Res. Comm., 1989, 164, 58

EP-629627-A

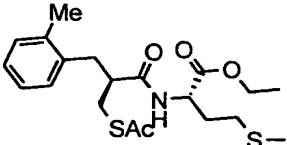
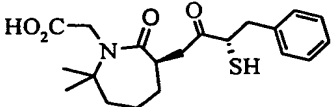
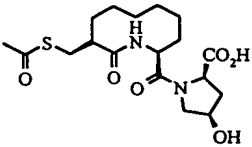
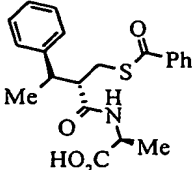
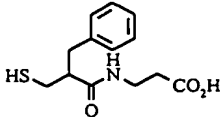
US-77978

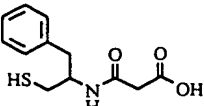
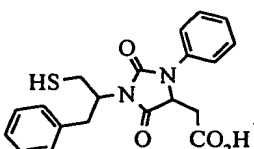
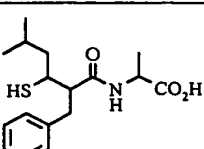
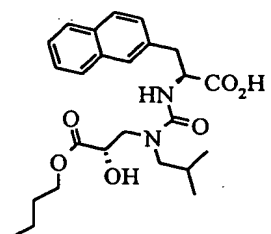
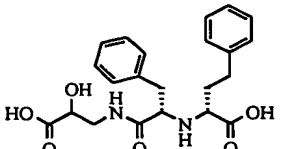
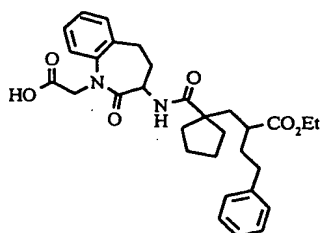
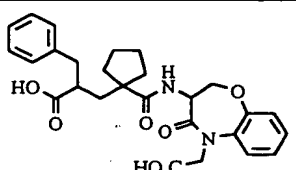
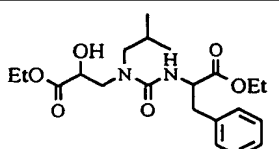
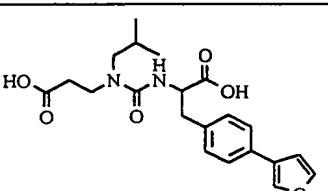
Perspect. Med. Chem. (1993), 45.

EP-358398-B

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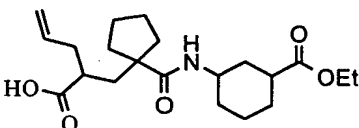
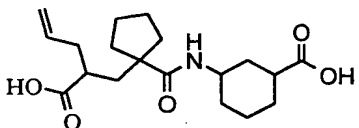
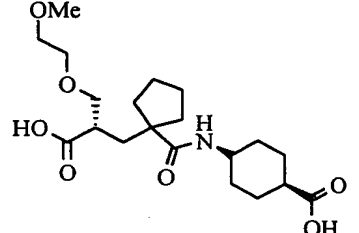
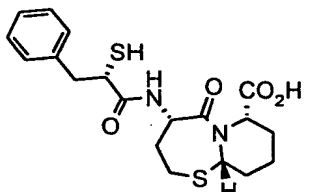
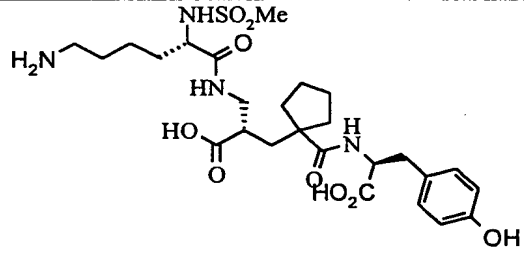
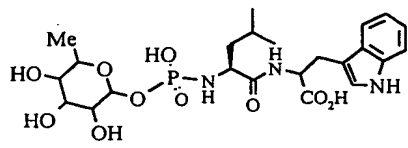
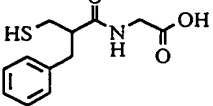
Further examples of I:NEPs are selected from the following structures:

<u>Compound</u>	<u>Structure</u>	<u>Mode of Action</u> <u>References</u>
FXII		I:NEP EP-509442A US-192435 US-4929641
FXIII		I:NEP (also an ACE inhibitor) EP-599444B US-884664
FXIV		I:NEP EP-544620A US-798684 J. Med. Chem. 1993, 3821.
FXV		I:NEP (also an ACE inhibitor) Mizanpril Circulation 1993, <u>88</u> (4), 1.
FXVI		I:NEP EP-136883 JP-85136554 US-4722810

FXVII		I:NEP Retrothiorphan Curr. Pharm. Design, 1996, 2, 443.
FXVIII		I:NEP (also an ACE inhibitor) EP-640594
FXIX		I:NEP J. Med. Chem. 1993, 36(1), 87.
FXX		I:NEP (also an ACE inhibitor) EP-738711-A JP-270957
FXXI		I:NEP CAS # 115406-23-0
FXXII		I:NEP (also an ECE inhibitor) DE-19510566 DE-19638020 EP-830863 JP-98101565
FXXIII		I:NEP (also an ECE inhibitor) EP-733642
FXXIV		I:NEP WO96/14293
FXXV		I:NEP JP-08245609 JP-96245609

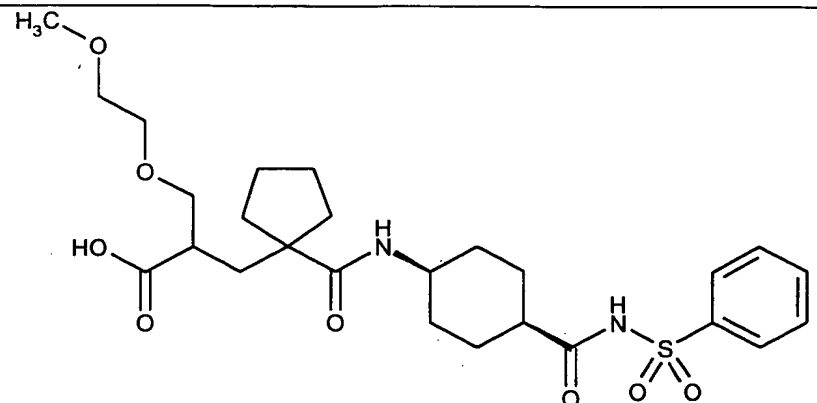
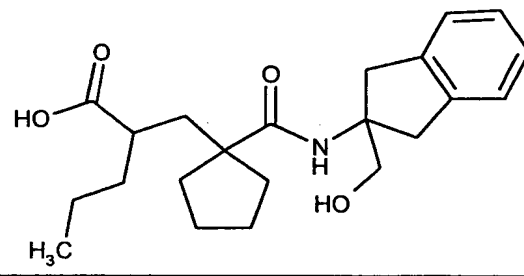
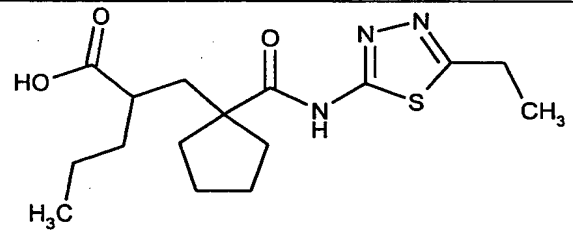
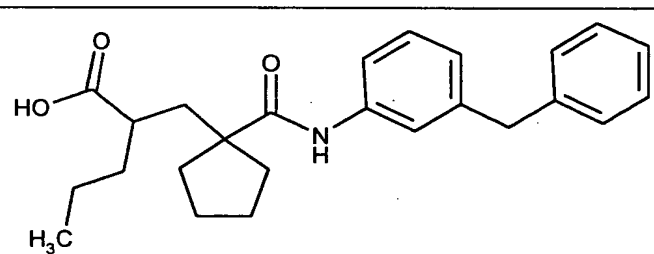
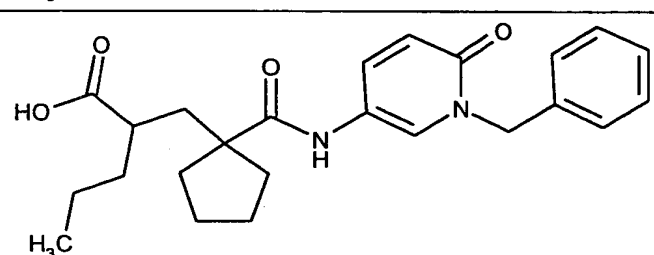
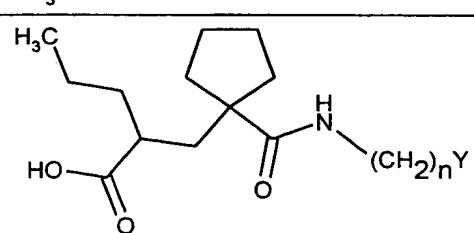
FXXVI		I:NEP WO9415908
FXXVII		I:NEP JP05092948
FXXVIII		I:NEP WO-9309101
FXXIX		I:NEP WO-9109840
FXXXI		I:NEP EP-519738 EP-690070
FXXXII		I:NEP (also an ACE inhibitor) J. Med. Chem. (1993), 36, 2420.
FXXXIII		I:NEP JP-95157459 Bioorg. Med. Chem. Letts., 1996, 6(1), 65.

Preferred additional I:NEPs are selected from the following structures:

<u>Compound</u>	<u>Structure</u>	<u>Mode f Action</u> <u>References</u>
FV		I:NEP EP-A-0274234 (Example 300)
FVI		I:NEP EP-A-0274234 (Example 379)
FVII		I:NEP Candoxatrilat EP-274234 JP-88165353 Biochem.Biophys.Res. Comm.,1989, <u>164</u> , 58
FVIII		I:NEP Omapatrilat (also an inhibitor of ACE) EP-0629627-A US-77978
FIX		I:NEP Sampatrilat (also an inhibitor of ACE) Perspect. Med. Chem. (1993), 45. EP-0358398-B
FX		I:NEP Phosphoramidon (which is commercially available)
FXI		I:NEP Thiorphan (which is commercially available)

More preferred additional I:NEPs are selected from the following structures:

COMPOUND	STRUCTURE
F57	 <chem>COCC(=O)N1CCCC1C(=O)N2C(=O)N(Cc3ccccc3)C=C2</chem>
F58	 <chem>O=C1NCCC1C(=O)N2CCCC2C(=O)N3CCCC3C(=O)N4CCCC4</chem>
F59	 <chem>OC1CCC2C(=O)N1C(=O)N3CCCC3C(=O)N4CCCC4C(=O)N5CCCC5</chem>
F60	 <chem>CC1=NC(=S)N=C1N2CCCC2C(=O)N3CCCC3C(=O)N4CCCC4C(=O)N5CCCC5</chem>

F61	
F62	
F63	
F64	
F65	
F66	 <p>(lc)</p>

These compounds were prepared according to the teachings presented in the Experimental section (*infra*). These compounds were tested as agents and were found to be useful for enhancing the endogenous erectile process, and thereby being useful in the treatment of MED.

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### Pharmaceutical Formulations

The compounds of the invention, their pharmaceutically acceptable salts, and pharmaceutically acceptable solvates of either entity can be administered alone but, in human therapy will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice.

For example, the compounds of the invention, or salts or solvates thereof can be administered orally, buccally or sublingually in the form of tablets, capsules (including soft gel capsules), ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed-, modified-, or controlled-release such as sustained-, dual-, or pulsatile delivery applications. The compounds of the invention may also be administered via intracavernosal injection. The compounds of the invention may also be administered via fast dispersing or fast dissolving dosage forms or in the form of a high energy dispersion or as coated particles. Suitable pharmaceutical formulations of the compounds of the invention may be in coated or un-coated form as desired.

Such tablets may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate, glycine and starch (preferably corn, potato or tapioca starch), disintegrants such as sodium starch glycollate, croscarmellose sodium and certain complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethyl cellulose (HPMC), hydroxypropylcellulose (HPC), sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules. Preferred excipients in this regard include lactose, starch, a cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions



and/or elixirs, the compounds of the invention may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

5

Modified release and pulsatile release dosage forms may contain excipients such as those detailed for immediate release dosage forms together with additional excipients that act as release rate modifiers, these being coated on and/or included in the body of the device. Release rate modifiers include, but are not exclusively limited to, hydroxypropylmethyl cellulose, methyl cellulose, sodium carboxymethylcellulose, ethyl cellulose, cellulose acetate, polyethylene oxide, Xanthan gum, Carbomer, ammonio methacrylate copolymer, hydrogenated castor oil, carnauba wax, paraffin wax, cellulose acetate phthalate, hydroxypropylmethyl cellulose phthalate, methacrylic acid copolymer and mixtures thereof. Modified release and pulsatile release dosage forms may contain one or a combination of release rate modifying excipients. Release rate modifying excipients maybe present both within the dosage form i.e. within the matrix, and/or on the dosage form i.e. upon the surface or coating.

Fast dispersing or dissolving dosage formulations (FDDFs) may contain the following ingredients: aspartame, acesulfame potassium, citric acid, croscarmellose sodium, crospovidone, diascorbic acid, ethyl acrylate, ethyl cellulose, gelatin, hydroxypropylmethyl cellulose, magnesium stearate, mannitol, methyl methacrylate, mint flavouring, polyethylene glycol, fumed silica, silicon dioxide, sodium starch glycolate, sodium stearyl fumarate, sorbitol, xylitol. The terms dispersing or dissolving as used herein to describe FDDFs are dependent upon the solubility of the drug substance used i.e. where the drug substance is insoluble a fast dispersing dosage form can be prepared and where the drug substance is soluble a fast dissolving dosage form can be prepared.

The compounds of the invention can also be administered parenterally, for example, intracavernosally, intravenously, intra-arterially, intraperitoneally, intrathecally, intraventricularly, intraurethrally intrasternally, intracranially, intramuscularly or subcutaneously, or they may be administered by infusion or needless injection techniques. For such parenteral administration they are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The

preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well-known to those skilled in the art.

- 5 For oral and parenteral administration to human patients, the daily dosage level of the compounds of the invention or salts or solvates thereof will usually be from 10 to 500 mg (in single or divided doses).

Thus, for example, tablets or capsules of the compounds of the invention or salts or solvates thereof may contain from 5 mg to 250 mg of active compound for  
10 administration singly or two or more at a time, as appropriate. The physician in any event will determine the actual dosage which will be most suitable for any individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be  
15 individual instances where higher or lower dosage ranges are merited and such are within the scope of this invention. The skilled person will also appreciate that, for in the treatment of MED according to the present invention, the NEPi (and where appropriate PDE5i or additional agents(s)) compounds may be taken as a single dose on an "as required" basis (i.e. as needed or desired).

20

#### Example Tablet Formulation

In general a tablet formulation could typically contain between about 0.01mg and 500mg of compound (or a salt thereof) whilst tablet fill weights may range from 50mg  
25 to 1000mg. An example formulation for a 10mg tablet is illustrated:

<u>Ingredient</u>	<u>%w/w</u>
Free acid, Free base or Salt of Compound	10.000*
Lactose	64.125
30 Starch	21.375
Croscarmellose Sodium	3.000
Magnesium Stearate	1.500

\* This quantity is typically adjusted in accordance with drug activity.

35

The tablets are manufactured by a standard process, for example, direct compression or a wet or dry granulation process. The tablet cores may be coated with appropriate overcoats.

5

The compounds / compositions can also be administered intranasally or by inhalation and are conveniently delivered in the form of a dry powder inhaler or an aerosol spray presentation from a pressurised container, pump, spray or nebuliser with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, 10 dichlorotetrafluoroethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFA 134A [trade mark] or 1,1,1,2,3,3,3-heptafluoropropane (HFA 227EA [trade mark])), carbon dioxide or other suitable gas. In the case of a pressurised aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurised container, pump, spray or nebuliser may contain a solution 15 or suspension of the active compound, e.g. using a mixture of ethanol and the propellant as the solvent, which may additionally contain a lubricant, e.g. sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of a compound of the invention and a suitable powder base such as lactose or starch.

20

Aerosol or dry powder formulations are preferably arranged so that each metered dose or "puff" contains from 1 to 50 mg of a compound of the invention for delivery to the patient. The overall daily dose with an aerosol will be in the range of from 1 to 50 mg which may be administered in a single dose or, more usually, in divided doses 25 throughout the day.

.5

The compounds also be formulated for delivery via an atomiser. Formulations for atomiser devices may contain the following ingredients as solubilisers, emulsifiers or suspending agents: water, ethanol, glycerol, propylene glycol, low molecular weight 30 polyethylene glycols, sodium chloride, fluorocarbons, polyethylene glycol ethers, sorbitan trioleate, oleic acid.

30

Alternatively, the compounds or salts or solvates thereof can be administered in the form of a suppository, or they may be applied topically in the form of a gel, hydrogel, 35 lotion, solution, cream, ointment or dusting powder. The compounds of the invention or salts or solvates thereof may also be dermally administered. The compounds of the invention or salts or solvates thereof may also be transdermally administered, for

35

example, by the use of a skin patch. They may also be administered by the ocular, pulmonary or rectal routes.

For ophthalmic use, the compounds can be formulated as micronised suspensions in isotonic, pH adjusted, sterile saline, or, preferably, as solutions in isotonic, pH adjusted, sterile saline, optionally in combination with a preservative such as a benzylalkonium chloride. Alternatively, they may be formulated in an ointment such as petrolatum.

- 10 For application topically to the skin, the compounds or salts or solvates thereof can be formulated as a suitable ointment containing the active compound suspended or dissolved in, for example, a mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, they can be
- 15 formulated as a suitable lotion or cream, suspended or dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan monostearate, a polyethylene glycol, liquid paraffin, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.
- 20 The compounds may also be used in combination with a cyclodextrin. Cyclodextrins are known to form inclusion and non-inclusion complexes with drug molecules. Formation of a drug-cyclodextrin complex may modify the solubility, dissolution rate, bioavailability and/or stability property of a drug molecule. Drug-cyclodextrin complexes are generally useful for most dosage forms and administration routes. As
- 25 an alternative to direct complexation with the drug the cyclodextrin may be used as an auxiliary additive, e.g. as a carrier, diluent or solubiliser. Alpha-, beta- and gamma-cyclodextrins are most commonly used and suitable examples are described in WO-A-91/11172, WO-A-94/02518 and WO-A-98/55148.
- 30 Generally, in humans, oral administration of the is the preferred route, being the most convenient in MED, avoiding the well-known disadvantages associated with intracavernosal (i.c.) administration. A preferred oral dosing regimen in MED for a typical man is from about 25mg to 500 mg of pharmaceutical composition when required. Where the composition comprises the combination of a NEPi and a PDE5I
- 35 then from 25mg to 250mg of each compound may be present. In circumstances where the recipient suffers from a swallowing disorder or from impairment of drug

absorption after oral administration, the drug may be administered parenterally, sublingually or buccally.

#### K<sub>i</sub> VALUES

5

For some applications, preferably the agent of the present invention (and optionally the optional additional agent) has a K<sub>i</sub> value of less than about 100 nM, preferably less than about 75 nM, preferably less than about 50 nM, preferably less than about 25 nM, preferably less than about 20 nM, preferably less than about 15 nM, preferably less than about 10 nM, preferably less than about 5 nM.

10

#### K<sub>b</sub> VALUES

15

For some applications, preferably the agent of the present invention (and optionally the optional additional agent) has a K<sub>b</sub> value of less than about 100 nM, preferably less than about 75 nM, preferably less than about 50 nM, preferably less than about 25 nM, preferably less than about 20 nM, preferably less than about 15 nM, preferably less than about 10 nM, preferably less than about 5 nM.

20

#### K<sub>a</sub> VALUES

For some applications, preferably the agent of the present invention (and optionally the optional additional agent) has a K<sub>a</sub> value of less than about 100 nM, preferably less than about 75 nM, preferably less than about 50 nM, preferably less than about 25 nM, preferably less than about 20 nM, preferably less than about 15 nM, preferably less than about 10 nM, preferably less than about 5 nM.

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#### PHARMACOKINETICS

For some embodiments of the present invention, preferably the NPY<sub>1</sub> agents for use in the treatment of MED according to the present invention (and optionally the optional additional agent) have a log D of -2 to +4, more preferably -1 to +2. The log D can be determined by standard procedures known in the art such as described in J. Pharm. Pharmacol. 1990, 42:144.

35

In addition, or in the alternative to the above, for some embodiments preferably the NPYi agents (and optionally the PDE5i and/or optional additional agent(s)) have a caco-2 flux of greater than  $2 \times 10^{-6} \text{cms}^{-1}$ , more preferably greater than  $5 \times 10^{-6} \text{cms}^{-1}$ . The caco flux value can be determined by standard procedures known in the art such as described in J. Pharm. Sci 79, 7, p595-600 (1990), and Pharm. Res. vol 14, no. 6 (1997).

### SELECTIVITY

10

For some applications, preferably the agent of the present invention (and optionally the optional additional agent) has at least about a 100 fold selectivity to the desired target, preferably at least about a 150 fold selectivity to the desired target, preferably at least about a 200 fold selectivity to the desired target, preferably at least about a 250 fold selectivity to the desired target, preferably at least about a 300 fold selectivity to the desired target, preferably at least about a 350 fold selectivity to the desired target.

15

For some applications, preferably the agent of the present invention (and optionally the optional additional agent) has at least about a 400 fold selectivity to the desired target, preferably at least about a 500 fold selectivity to the desired target, preferably at least about a 600 fold selectivity to the desired target, preferably at least about a 700 fold selectivity to the desired target, preferably at least about a 800 fold selectivity to the desired target, preferably at least about a 900 fold selectivity to the desired target, preferably at least about a 1000 fold selectivity to the desired target.

20

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### BIOAVAILABILITY

To be effective as a treatment, the compounds of the invention should be orally bioavailable. Oral bioavailability refers to the proportion of an orally administered drug that reaches the systemic circulation. The factors that determine oral bioavailability of a drug are dissolution, membrane permeability and metabolic stability. Typically, a screening cascade of firstly *in vitro* and then *in vivo* techniques is used to determine oral bioavailability.

30

35

Dissolution, the solubilisation of the drug by the aqueous contents of the gastrointestinal tract (GIT), can be predicted from *in vitro* solubility experiments conducted at appropriate pH to mimic the GIT. Preferably the compounds of the invention have a minimum solubility of 50 mcg/ml. Solubility can be determined by standard procedures known in the art such as described in Adv. Drug Deliv. Rev. 23, 3-25, 1997.

Membrane permeability refers to the passage of the compound through the cells of the GIT. Lipophilicity is a key property in predicting this and is defined by *in vitro* Log  $D_{7.4}$  measurements using organic solvents and buffer. Preferably the compounds of the invention have a Log  $D_{7.4}$  of -2 to +4, more preferably -1 to +2. The log D can be determined by standard procedures known in the art such as described in J. Pharm. Pharmacol. 1990, 42:144.

Cell monolayer assays such as CaCo<sub>2</sub> add substantially to prediction of favourable membrane permeability in the presence of efflux transporters such as p-glycoprotein, so-called caco-2 flux. Preferably, compounds of the invention have a caco-2 flux of greater than  $2 \times 10^{-6} \text{cms}^{-1}$ , more preferably greater than  $5 \times 10^{-6} \text{cms}^{-1}$ . The caco flux value can be determined by standard procedures known in the art such as described in J. Pharm. Sci, 1990, 79, 595-600.

Metabolic stability addresses the ability of the GIT or the liver to metabolise compounds during the absorption process: the first pass effect. Assay systems such as microsomes, hepatocytes etc are predictive of metabolic liability. Preferably the compounds of the Examples show metabolic stability in the assay system that is commensurate with an hepatic extraction of less than 0.5. Examples of assay systems and data manipulation are described in Curr. Opin. Drug Disc. Devel., 201, 4, 36-44, Drug Met. Disp., 2000, 28, 1518-1523.

Because of the interplay of the above processes further support that a drug will be orally bioavailable in humans can be gained by *in vivo* experiments in animals. Absolute bioavailability is determined in these studies by administering the compound separately or in mixtures by the oral route. For absolute determinations (% absorbed) the intravenous route is also employed. Examples of the assessment of oral bioavailability in animals can be found in Drug Met. Disp., 2001, 29, 82-87; J. Med Chem, 1997, 40, 827-829, Drug Met. Disp., 1999, 27, 221-226.

## CHEMICAL SYNTHESIS METHODS

Typically the NPYi and PDE5i compounds suitable for the use according to the present invention will be prepared by chemical synthesis techniques.

5

The agent or target or variants, homologues, derivatives, fragments or mimetics thereof may be produced using chemical methods to synthesize the agent in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography (e.g., Creighton (1983) *Proteins Structures And Molecular Principles*, WH Freeman and Co, New York NY). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton, *supra*).

10

Direct synthesis of the agent or variants, homologues, derivatives, fragments or mimetics thereof can be performed using various solid-phase techniques (Roberge JY *et al* (1995) *Science* 269: 202-204) and automated synthesis may be achieved, for example, using the ABI 431A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer. Additionally, the amino acid sequences comprising the agent or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with a sequence from other subunits, or any part thereof, to produce a variant agent or target, such as, for example, a variant NEP.

20

In an alternative embodiment of the invention, the coding sequence of the agent target or variants, homologues, derivatives, fragments or mimetics thereof may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al* (1980) *Nuc Acids Res Symp Ser* 215-23, Horn T *et al* (1980) *Nuc Acids Res Symp Ser* 225-232).

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## MIMETIC

As used herein, the term "mimetic" relates to any chemical which includes, but is not limited to, a peptide, polypeptide, antibody or other organic chemical which has the same qualitative activity or effect as a reference agent to a target.

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### CHEMICAL DERIVATIVE

The term "derivative" or "derivatised" as used herein includes chemical modification of an agent. Illustrative of such chemical modifications would be replacement of  
5 hydrogen by a halo group, an alkyl group, an acyl group or an amino group.

### CHEMICAL MODIFICATION

In one embodiment of the present invention, the agent may be a chemically modified  
10 agent.

The chemical modification of an agent may either enhance or reduce hydrogen bonding interaction, charge interaction, hydrophobic interaction, Van Der Waals interaction or dipole interaction between the agent and the target.  
15

In one aspect, the identified agent may act as a model (for example, a template) for the development of other compounds.

### RECOMBINANT METHODS

20 Typically the target for use in the assay of the present invention may be prepared by recombinant DNA techniques.

### POTENTIATING cGMP

As used herein with reference to cGMP, the term "potentiating" includes any one or more of: increasing the effectiveness of cGMP, increasing the levels of cGMP, increasing the activity of cGMP, decreasing the level of cGMP degradation,  
30 decreasing the level of cGMP inhibition.

The potentiating effect can be a direct effect. Alternatively, it could be a secondary effect and/or a downstream effect.

35 Here, preferably, the agent that potentiates cGMP acts on a  $I_{cGMP}$  and/or an  $AM_{cGMP}$  wherein the modulator of cGMP has an adverse effect on cGMP, such that the agent

reduces and/or eliminates and/or masks and/or diverts the detrimental effect of the  $I_{cGMP}$  and/or the  $AM_{cGMP}$  towards cGMP.

Hence, the present invention encompasses a combination of one or more  $I:I_{cAMP}$  and  
5 one or more  $I:I_{cGMP}$ . In one aspect, the  $I:I_{cGMP}$  is a  $I:PDE_{cGMP}$ .

$I_{cAMP}$  AND/OR  $AM_{cAMP}$

10 We have shown that cAMP mediates male genital blood flow and by enhancing cAMP signalling we can enhance male genital blood flow in an animal model. Thus, an agent that upregulates/enhances cAMP-mediated vasorelaxation will be efficacious in the treatment of MED. For ease of reference, we refer to these substances as  $I_{cAMP}$  and/or an  $AM_{cAMP}$ . Here, the  $I_{cAMP}$  and the  $AM_{cAMP}$  have an  
15 adverse effect on cAMP levels or activity.

Thus, the agent may be any one of more of: an  $I:I_{cAMP}$  and/or an  $I:AM_{cAMP}$ .

The agent may be a single entity that is capable of exhibiting two or more of these  
20 properties. Alternatively, or in addition, the agent can be a combination of agents that are capable of exhibiting one or more of these properties.

Examples of  $I_{cAMP}$  and the  $AM_{cAMP}$  include NPY and optionally one or more of PDE(s) and NEP, or any component associated therewith. The associated component may  
.5 be, for example, a receptor and/or a co-factor.

Thus, preferably the agent of the present invention may be used in conjunction with any one of more of: an  $I:PDE_{cAMP}$ , and  $I:NEP$ .

30 Likewise, the agent may be a single entity that is capable of exhibiting two or more of these properties. Alternatively, or in addition, the agent can be a combination of agents that are capable of exhibiting one or more of these properties.

$I:I_{cAMP}$  AND/OR  $I:AM_{cAMP}$

35

In accordance with the present invention we have found that it is possible to treat and/or prevent MED by using an agent that reduces and/or eliminates and/or masks

and/or diverts and/or prevents the detrimental effect of the  $I_{cAMP}$  and/or the  $AM_{cAMP}$  towards cAMP. The agent may even restore cAMP levels that were decreased by the  $I_{cAMP}$  and/or a  $AM_{cAMP}$ . For ease, we refer to these substances as  $I:I_{cAMP}$  and/or a  $I:AM_{cAMP}$ . Here, the  $I:I_{cAMP}$  and the  $I:AM_{cAMP}$  prevent or reduce the adverse effect on cAMP levels or activity.

Thus, in one preferred aspect, the agent is an  $I:I_{cAMP}$  and/or an  $I:AM_{cAMP}$  wherein the  $AM_{cAMP}$  has a detrimental effect on  $AM_{cAMP}$ .

10  $A_{cAMP}$

In accordance with the present invention, we have found that one of the important causes of MED is due to low levels or low activity of cAMP in the male genitalia.

15 Thus, the agent may be a  $U:A_{cAMP}$ .

Thus, preferably the agent of the present invention may also be able to act as, and/or may be used in conjunction with, any one of more of:  $A:AC$ ,  $A:VIP_r$ ,  $A:VIP_n$ ,  $I:I:VIP_r$  or  $I:I:VIP_n$ .

20

The agent may be a single entity that is capable of exhibiting two or more of these properties. Alternatively, or in addition, the agent can be a combination of agents that are capable of exhibiting one or more of these properties.

25  $U:A_{cAMP}$

In another respect, an additional target may be a component that increases the level of cAMP. Hence, the agent can also act as an  $U:AC$

30 Hence, by way of example, the agent of the present invention may also be able to act as, and/or may be used in conjunction with, any one of more of agent can be any one of: an  $U:A_{cAMP}$ , an  $A:AC$ , an  $A:VIP_r$ , an  $A:VIP_n$ , an  $I:I:VIP_r$  or an  $I:I:VIP_n$ .

35 By way of example, the target could be cAMP itself or AC or VIP (or combinations thereof).

COMBINATION OF  $I:I_{cAMP}$  AND/OR  $I:M_{cAMP}$  AND/OR  $U:A_{cAMP}$

In another aspect, the agent of the present invention may be used with a combination of cAMP potentiators. By way of example, the agent of the present invention may be used in combination with one or more of:

I:PDE<sub>cAMP</sub>

I:PDE<sub>n</sub><sub>cAMP</sub>

I:NPY

I:NPY Y<sub>n</sub>

I:NEP

U:A<sub>cAMP</sub>

A:AC

A:VIP<sub>r</sub>

A:VIP<sub>n</sub>

I:I:VIP<sub>r</sub>

I:I:VIP<sub>n</sub>.

CAMP mimetic

### INHIBITOR

The term "inhibitor" as used herein with respect to the agent of the present invention means an agent that can reduce and/or eliminate and/or mask and/or prevent the detrimental action of a I<sub>cAMP</sub> and/or a detrimental M<sub>cAMP</sub> towards cAMP. The inhibitor may act as an antagonist.

### ACTIVATOR

The term "activator" as used herein with respect to the agent of the present invention means an agent that can increase and/or produce and/or unmask and/or elevate and/or ensure action of cAMP and/or an A<sub>cAMP</sub>. The activator may act as an agonist.

### OTHER ACTIVE COMPONENTS

In another aspect, the agent of the present invention may even be in combination with one or more other active components - such as one or more agents capable of potentiating cGMP.

### AMINO ACID SEQUENCE

As used herein, the term "amino acid sequence" is synonymous with the term  
5 "polypeptide" and/or the term "protein". In some instances, the term "amino acid  
sequence" is synonymous with the term "peptide". In some instances, the term  
"amino acid sequence" is synonymous with the term "protein".

The amino acid sequence may be prepared isolated from a suitable source, or it may  
10 be made synthetically or it may be prepared by use of recombinant DNA techniques.

### NUCLEOTIDE SEQUENCE

15 As used herein, the term "nucleotide sequence" is synonymous with the term  
"polynucleotide".

The nucleotide sequence may be DNA or RNA of genomic or synthetic or of  
recombinant origin. The nucleotide sequence may be double-stranded or single-  
20 stranded whether representing the sense or antisense strand or combinations  
thereof.

For some applications, preferably, the nucleotide sequence is DNA.

25 For some applications, preferably, the nucleotide sequence is prepared by use of  
recombinant DNA techniques (e.g. recombinant DNA).

For some applications, preferably, the nucleotide sequence is cDNA.

30 For some applications, preferably, the nucleotide sequence may be the same as the  
naturally occurring form for this aspect.

It will be understood by a skilled person that numerous different nucleotide  
sequences can encode the targets as a result of the degeneracy of the genetic code.  
35 In addition, it is to be understood that skilled persons may, using routine techniques,  
make nucleotide substitutions that do not substantially affect the activity encoded by the  
nucleotide sequence of the present invention to reflect the codon usage of any

particular host organism in which the target is to be expressed. Thus, the terms "variant", "homologue" or "derivative" in relation to the nucleotide sequence set out in the attached sequence listings include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence encodes a functional target according the present invention (or even an agent according to the present invention if said agent comprises a nucleotide sequence or an amino acid sequence).

As indicated above, with respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequences shown in the sequence listing herein. More preferably there is at least 95%, more preferably at least 98%, homology. Nucleotide homology comparisons may be conducted as described above. A preferred sequence comparison program is the GCG Wisconsin Bestfit program described above. The default scoring matrix has a match value of 10 for each identical nucleotide and -9 for each mismatch. The default gap creation penalty is -50 and the default gap extension penalty is -3 for each nucleotide.

The present invention also encompasses nucleotide sequences that are capable of hybridising selectively to the sequences presented herein, or any variant, fragment or derivative thereof, or to the complement of any of the above. Nucleotide sequences are preferably at least 15 nucleotides in length, more preferably at least 20, 30, 40 or 50 nucleotides in length. These sequences could be used as probes, such as in a diagnostic kit.

#### VARIANTS/HOMOLOGUES/DERIVATIVES

In addition to the specific amino acid sequences and nucleotide sequences mentioned herein, the present invention also encompasses the use of variants, homologue and derivatives thereof. Here, the term "homology" can be equated with "identity".

In the present context, an homologous sequence is taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical. In particular, homology should typically be considered with respect to those regions of the sequence known to be essential for an activity. Although homology can also be considered in terms of similarity (i.e. amino acid residues

having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of  
5 readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly  
10 compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration  
15 that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without  
20 penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence  
5 alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce  
30 optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

35

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer

program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 5 18), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410) and the GENWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see FEMS Microbiol Lett 10 1999 174(2): 247-50; FEMS Microbiol Lett 1999 177(1): 187-8 and tatiana@ncbi.nlm.nih.gov).

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a 15 scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for 20 further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of 5 the sequence comparison and generates a numerical result.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of 30 similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity 35 values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.



Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	GAP
		ILV
	Polar - uncharged	CSTM
		NQ
	Polar - charged	DE
		KR
AROMATIC		HFVY

5

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

15

Replacements may also be made by unnatural amino acids include; alpha\* and alpha-disubstituted\* amino acids, N-alkyl amino acids\*, lactic acid\*, halide derivatives of natural amino acids such as trifluorotyrosine\*, p-Cl-phenylalanine\*, p-Br-phenylalanine\*, p-I-phenylalanine\*, L-allyl-glycine\*,  $\beta$ -alanine\*, L- $\alpha$ -amino butyric acid\*, L- $\gamma$ -amino butyric acid\*, L- $\alpha$ -amino isobutyric acid\*, L- $\epsilon$ -amino caproic acid<sup>#</sup>, 7-amino heptanoic acid\*, L-methionine sulfone<sup>#</sup>, L-norleucine\*, L-norvaline\*, p-nitro-L-phenylalanine\*, L-hydroxyproline<sup>#</sup>, L-thioprolin<sup>#</sup>, methyl derivatives of phenylalanine (Phe) such as 4-methyl-Phe\*, pentamethyl-Phe\*, L-Phe (4-amino)<sup>#</sup>, L-Tyr (methyl)\*, L-Phe (4-isopropyl)\*, L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid)\*, L-diaminopropionic acid<sup>#</sup> and L-Phe (4-benzyl)\*. The notation \* has been utilised for the purpose of the discussion above (relating to homologous or non-homologous substitution), to indicate the hydrophobic nature of the derivative whereas # has been utilised to indicate the hydrophilic nature of the derivative, #\* indicates amphipathic characteristics.

30

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or  $\beta$ -alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the  $\alpha$ -carbon substituent group is on the residue's nitrogen atom rather than the  $\alpha$ -carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., PNAS (1992) 89(20), 9367-9371 and Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134.

### HYBRIDISATION

The present invention also encompasses the use of sequences that can hybridise to the target sequences presented herein – such as if the agent is an anti-sense sequence.

The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies.

Nucleotide sequences of the invention capable of selectively hybridising to the nucleotide sequences presented herein, or to their complement, will be generally at least 75%, preferably at least 85 or 90% and more preferably at least 95% or 98% homologous to the corresponding complementary nucleotide sequences presented herein over a region of at least 20, preferably at least 25 or 30, for instance at least 40, 60 or 100 or more contiguous nucleotides. Preferred nucleotide sequences of the invention will comprise regions homologous to the nucleotide sequence set out in SEQ ID No 2 of the sequence listings of the present invention preferably at least 80 or 90% and more preferably at least 95% homologous to the nucleotide sequence set out in SEQ ID No 2 of the sequence listings of the present invention.

The term "selectively hybridizable" means that the nucleotide sequence, when used as a probe, is used under conditions where a target nucleotide sequence is found to hybridize to the probe at a level significantly above background. The background

hybridization may occur because of other nucleotide sequences present, for example, in the cDNA or genomic DNA library being screened. In this event, background implies a level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10 fold, preferably less than 100 fold as intense as the specific interaction observed with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with  $^{32}\text{P}$ .

Hybridization conditions are based on the melting temperature ( $T_m$ ) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about  $T_m - 5^\circ\text{C}$  ( $5^\circ\text{C}$  below the  $T_m$  of the probe); high stringency at about  $5^\circ\text{C}$  to  $10^\circ\text{C}$  below  $T_m$ ; intermediate stringency at about  $10^\circ\text{C}$  to  $20^\circ\text{C}$  below  $T_m$ ; and low stringency at about  $20^\circ\text{C}$  to  $25^\circ\text{C}$  below  $T_m$ . As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related polynucleotide sequences.

20

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention under stringent conditions (e.g.  $65^\circ\text{C}$  and  $0.1\times\text{SSC}$  { $1\times\text{SSC} = 0.15\text{ M NaCl}$ ,  $0.015\text{ M Na}_3\text{ Citrate pH } 7.0$ ). Where the nucleotide sequence of the invention is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the present invention. Where the nucleotide sequence is single-stranded, it is to be understood that the complementary sequence of that nucleotide sequence is also included within the scope of the present invention.

Nucleotide sequences which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of sources. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising

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to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of the nucleotide sequence set out in herein under conditions of medium to high stringency.

5 Similar considerations apply to obtaining species homologues and allelic variants of the amino acid and/or nucleotide sequences of the present invention.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and  
10 homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used. The primers used in degenerate PCR will  
15 contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such nucleotide sequences may be obtained by site directed mutagenesis  
20 of characterised sequences, such as the nucleotide sequence set out in SEQ ID No 2 of the sequence listings of the present invention. This may be useful where for example silent codon changes are required to sequences to optimise codon preferences for a particular host cell in which the nucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition  
25 sites, or to alter the activity of the protein encoded by the nucleotide sequences.

The nucleotide sequences of the present invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-  
30 radioactive labels, or the nucleotide sequences may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term nucleotide sequence of the invention as used herein.

35 The nucleotide sequences such as a DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

5

Longer nucleotide sequences will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction (PCR) under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

10

15

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used to clone and express the target sequences. As will be understood by those of skill in the art, for certain expression systems, it may be advantageous to produce the target sequences with non-naturally occurring codons. Codons preferred by a particular prokaryotic or eukaryotic host (Murray E *et al* (1989) Nuc Acids Res 17:477-508) can be selected, for example, to increase the rate of the target expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

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### EXPRESSION VECTORS

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The nucleotide sequence for use as the target or for expressing the target can be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence in and/or from a compatible host cell. Expression may be controlled using control sequences which include promoters/enhancers and other expression regulation signals. Prokaryotic promoters and promoters functional in eukaryotic cells may be used. Tissue specific or stimuli

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specific promoters may be used. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

The protein produced by a host recombinant cell by expression of the nucleotide sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences can be designed with signal sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

## 10 FUSION PROTEINS

The target amino acid sequence may be produced as a fusion protein, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and  $\beta$ -galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the target.

20 The fusion protein may comprise an antigen or an antigenic determinant fused to the substance of the present invention. In this embodiment, the fusion protein may be a non-naturally occurring fusion protein comprising a substance which may act as an adjuvant in the sense of providing a generalised stimulation of the immune system. The antigen or antigenic determinant may be attached to either the amino or carboxy terminus of the substance.

In another embodiment of the invention, the amino acid sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognized by a commercially available antibody.

## ANTIBODIES

35 In one embodiment of the present invention, the agent may be an antibody. In addition, or in the alternative, the target may be an antibody. In addition, or in the alternative, the means for detecting the target may be an antibody.

Antibodies may be produced by standard techniques, such as by immunisation with the substance of the invention or by using a phage display library.

5 For the purposes of this invention, the term "antibody", unless specified to the contrary, includes but is not limited to, polyclonal, monoclonal, chimeric, single chain, Fab fragments, fragments produced by a Fab expression library, as well as mimetics thereof. Such fragments include fragments of whole antibodies which retain their binding activity for a target substance, Fv, F(ab') and F(ab')<sub>2</sub> fragments, as well as single  
10 chain antibodies (scFv), fusion proteins and other synthetic proteins which comprise the antigen-binding site of the antibody. Furthermore, the antibodies and fragments thereof may be humanised antibodies. Neutralizing antibodies, i.e., those which inhibit biological activity of the substance polypeptides, are especially preferred for diagnostics and therapeutics.

15 If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide bearing a epitope(s) obtainable from an identified agent and/or substance of the present invention. Depending on the host species, various adjuvants may be used to increase  
20 immunological response. Such adjuvants include, but are not limited to, Freund's, mineral gels such as aluminium hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. BCG (*Bacilli Calmette-Guerin*) and *Corynebacterium parvum* are potentially useful human adjuvants which may be employed if purified the  
25 substance polypeptide is administered to immunologically compromised individuals for the purpose of stimulating systemic defence.

Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies to an epitope obtainable from  
30 an identified agent and/or substance of the present invention contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides polypeptides of the invention or fragments thereof haptenised to another  
35 polypeptide for use as immunogens in animals or humans.

Monoclonal antibodies directed against epitopes obtainable from an identified agent and/or substance of the present invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against orbit epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

Monoclonal antibodies to the substance and/or identified agent may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (1975 *Nature* 256:495-497), the human B-cell hybridoma technique (Kosbor *et al* (1983) *Immunol Today* 4:72; Cote *et al* (1983) *Proc Natl Acad Sci* 80:2026-2030) and the EBV-hybridoma technique (Cole *et al* (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R Liss Inc, pp 77-96). In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used (Morrison *et al* (1984) *Proc Natl Acad Sci* 81:6851-6855; Neuberger *et al* (1984) *Nature* 312:604-608; Takeda *et al* (1985) *Nature* 314:452-454). Alternatively, techniques described for the production of single chain antibodies (US Patent No. 4,946,779) can be adapted to produce the substance specific single chain antibodies.

Antibodies, both monoclonal and polyclonal, which are directed against epitopes obtainable from an identified agent and/or substance are particularly useful in diagnosis, and those which are neutralising are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotypic antibodies. Anti-idiotypic antibodies are immunoglobulins which carry an "internal image" of the substance and/or agent against which protection is desired. Techniques for raising anti-idiotypic antibodies are known in the art. These anti-idiotypic antibodies may also be useful in therapy.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi *et al* (1989, *Proc Natl Acad Sci* 86: 3833-3837), and Winter G and Milstein C (1991; *Nature* 349:293-299).



Antibody fragments which contain specific binding sites for the substance may also be generated. For example, such fragments include, but are not limited to, the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse WD *et al* (1989) Science 256:1275-128 1).

## REPORTERS

A wide variety of reporters may be used in the assay methods (as well as screens) of the present invention with preferred reporters providing conveniently detectable signals (eg. by spectroscopy). By way of example, a reporter gene may encode an enzyme which catalyses a reaction which alters light absorption properties.

Examples of reporter molecules include but are not limited to  $\beta$ -galactosidase, invertase, green fluorescent protein, luciferase, chloramphenicol, acetyltransferase,  $\beta$ -glucuronidase, exo-glucanase and glucoamylase. Alternatively, radiolabelled or fluorescent tag-labelled nucleotides can be incorporated into nascent transcripts which are then identified when bound to oligonucleotide probes.

In one preferred embodiment, the production of the reporter molecule is measured by the enzymatic activity of the reporter gene product, such as  $\beta$ -galactosidase.

A variety of protocols for detecting and measuring the expression of the target, such as by using either polyclonal or monoclonal antibodies specific for the protein, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilising monoclonal antibodies reactive to two non-interfering epitopes on polypeptides is preferred, but a competitive binding assay may be employed. These and other assays are described, among other places, in Hampton R *et al* (1990, Serological Methods, A Laboratory Manual, APS Press, St Paul MN) and Maddox DE *et al* (1983, J Exp Med 15 8:121 1).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays. Means for producing labelled hybridisation or PCR probes for detecting the target polynucleotide sequences include oligolabelling, nick translation, end-labelling or PCR amplification using a labelled nucleotide. Alternatively, the coding sequence, or any portion of it, may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labelled nucleotides.

A number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3817837; US-A-3850752; US-A-3939350; US-A-3996345; US-A-4277437; US-A-4275149 and US-A-4366241. Also, recombinant immunoglobulins may be produced as shown in US-A-4816567.

Additional methods to quantify the expression of a particular molecule include radiolabeling (Melby PC *et al* 1993 J Immunol Methods 159:235-44) or biotinylating (Duplaa C *et al* 1993 Anal Biochem 229:36) nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated. Quantification of multiple samples may be speeded up by running the assay in an ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or calorimetric response gives rapid quantification.

Although the presence/absence of marker gene expression suggests that the gene of interest is also present, its presence and expression should be confirmed. For example, if the nucleotide sequence is inserted within a marker gene sequence, recombinant cells containing the same may be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a target coding sequence under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the target as well.

Alternatively, host cells which contain the coding sequence for the target and express the target coding regions may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridisation and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

#### GENERAL ASSAYS FOR cAMP ACTIVITY/LEVELS

The ability of a test agent to potentiate cAMP may be determined by measuring a relevant increase or decrease of a target level. In addition, or in the alternative, the ability of a test agent to potentiate cAMP may be determined by measuring a relevant increase in cAMP levels. By way of example, one may adapt the teachings of Smith *et al* 1993 (Appl. Biochem. Biotechnol. 41:189-218). There are also commercially available immunoassay kits for the measurement of cAMP (eg Amersham International, Arlington Heights, IL and DuPont, Boston, MA). Details on a suitable cAMP assay are provided in the Experimental Section.

#### SCREENS

Any one or more of appropriate targets - such as an amino acid sequence and/or nucleotide sequence - may be used for identifying a NEPi in any of a variety of drug screening techniques. The target employed in such a test may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The target may even be within an animal model, wherein said target may be an exogenous target or an introduced target. The animal model will be a non-human animal model. The abolition of target activity or the formation of binding complexes between the target and the agent being tested may be measured.

Techniques for drug screening may be based on the method described in Geysen, European Patent Application 84/03564, published on September 13, 1984. In summary, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with a suitable target or fragment thereof and washed.

Bound entities are then detected - such as by appropriately adapting methods well known in the art. A purified target can also be coated directly onto plates for use in a drug screening techniques. Alternatively, non-neutralising antibodies can be used to capture the peptide and immobilise it on a solid support.

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This invention also contemplates the use of competitive drug screening assays in which neutralising antibodies capable of binding a target specifically compete with a test compound for binding to a target.

10 Another technique for screening provides for high throughput screening (HTS) of agents having suitable binding affinity to the substances and is based upon the method described in detail in WO 84/03564.

15 It is expected that the assay methods of the present invention will be suitable for both small and large-scale screening of test compounds as well as in quantitative assays.

Thus, the present invention also relates to a method of identifying agents that potentiate cAMP, the method comprising contacting a suitable target with the agent and then measuring the activity and/or levels of cAMP.

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The present invention also relates to a method of identifying agents that selectively potentiate cAMP in male sexual genitalia, the method comprising contacting a suitable target from male sexual genitalia and then measuring the activity and/or levels of cAMP.

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The present invention also relates to a method of identifying agents that potentiate cAMP, the method comprising contacting a suitable target with the agent and then measuring the activity and/or levels of the target.

30 The present invention also relates to a method of identifying agents that selectively potentiate cAMP in male sexual genitalia, the method comprising contacting a suitable target from male sexual genitalia and then measuring the activity and/or levels of the target.

35 In a preferred aspect, the screen of the present invention comprises at least the following steps (which need not be in this same consecutive order): (a) conducting an *in vitro* screen to determine whether a candidate agent has the relevant activity; (b)

conducting one or more selectivity screens to determine the selectivity of said candidate agent; and (c) conducting an *in vivo* screen with said candidate agent (e.g. using a functional animal model). Typically, if said candidate agent passes screen (a) and screen (b) then screen (c) is performed.

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## DIAGNOSTICS

The present invention also provides a diagnostic composition or kit for the detection of a pre-disposition for MED. In this respect, the composition or kit will comprise an entity that is capable of indicating the presence of one or more - or even the absence of one or more - of the targets in a test sample. Preferably, the test sample is obtained from the penis.

By way of example, the diagnostic composition may comprise any one of the nucleotide sequences mentioned herein or a variant, homologue, fragment or derivative thereof, or a sequence capable of hybridising to all or part of any one of the nucleotide sequence.

In order to provide a basis for the diagnosis of disease, normal or standard values from a target should be established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with an antibody to a target under conditions suitable for complex formation which are well known in the art. The amount of standard complex formation may be quantified by comparing it to a dilution series of positive controls where a known amount of antibody is combined with known concentrations of a purified target. Then, standard values obtained from normal samples may be compared with values obtained from samples from subjects potentially affected by MED. Deviation between standard and subject values establishes the presence of the disease state.

A target itself, or any part thereof, may provide the basis for a diagnostic and/or a therapeutic compound. For diagnostic purposes, target polynucleotide sequences may be used to detect and quantify gene expression in conditions, disorders or diseases in which MED may be implicated.

The target encoding polynucleotide sequence may be used for the diagnosis of MED resulting from expression of the target. For example, polynucleotide sequences encoding a target may be used in hybridisation or PCR assays of tissues from

biopsies or autopsies or biological fluids, to detect abnormalities in target expression. The form of such qualitative or quantitative methods may include Southern or northern analysis, dot blot or other membrane-based technologies; PCR technologies; dip stick, pin or chip technologies; and ELISA or other multiple sample  
5 formal technologies. All of these techniques are well known in the art and are in fact the basis of many commercially available diagnostic kits.

Such assays may be tailored to evaluate the efficacy of a particular therapeutic treatment regime and may be used in animal studies, in clinical trials, or in monitoring  
10 the treatment of an individual patient. In order to provide a basis for the diagnosis of disease, a normal or standard profile for target expression should be established. This is accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with the target or a portion thereof, under conditions suitable for hybridisation or amplification. Standard hybridisation may be  
15 quantified by comparing the values obtained for normal subjects with a dilution series of positive controls run in the same experiment where a known amount of purified target is used. Standard values obtained from normal samples may be compared with values obtained from samples from subjects potentially affected by a disorder or disease related to expression of the target coding sequence. Deviation between  
20 standard and subject values establishes the presence of the disease state. If disease is established, an existing therapeutic agent is administered, and treatment profile or values may be generated. Finally, the assay may be repeated on a regular basis to evaluate whether the values progress toward or return to the normal or standard pattern. Successive treatment profiles may be used to show the efficacy of  
25 treatment over a period of several days or several months.

Thus, in one aspect, the present invention relates to the use of a target polypeptide, or variant, homologue, fragment or derivative thereof, to produce anti-target antibodies which can, for example, be used diagnostically to detect and quantify  
30 target levels in MED.

The present invention further provides diagnostic assays and kits for the detection of a target in cells and tissues comprising a purified target which may be used as a positive control, and anti-target antibodies. Such antibodies may be used in solution-  
35 based, membrane-based, or tissue-based technologies to detect any disease state or condition related to the expression of target protein or expression of deletions or a variant, homologue, fragment or derivative thereof.

## ASSAY METHODS

The diagnostic compositions and/or methods and/or kits may be used in the following techniques which include but are not limited to; competitive and non-competitive assays, radioimmunoassay, bioluminescence and chemiluminescence assays, fluorometric assays, sandwich assays, immunoradiometric assays, dot blots, enzyme linked assays including ELISA, microtiter plates, antibody coated strips or dipsticks for rapid monitoring of urine or blood, immunohistochemistry and immunocytochemistry.

By way of example, an immunohistochemistry kit may also be used for localization of NPY activity in genital tissue. This immunohistochemistry kit permits localization of NPI in tissue sections and cultured cells using both light and electron microscopy which may be used for both research and clinical purposes. Such information may be useful for diagnostic and possibly therapeutic purposes in the detection and/or prevention and/or treatment of MED. For each kit the range, sensitivity, precision, reliability, specificity and reproducibility of the assay are established. Intraassay and interassay variation is established at 20%, 50% and 80% points on the standard curves of displacement or activity.

## PROBES

Another aspect of the subject invention is the provision of nucleic acid hybridisation or PCR probes which are capable of detecting (especially those that are capable of selectively selecting) polynucleotide sequences, including genomic sequences, encoding a target coding region or closely related molecules, such as alleles. The specificity of the probe, i.e., whether it is derived from a highly conserved, conserved or non-conserved region or domain, and the stringency of the hybridisation or amplification (high, intermediate or low) will determine whether the probe identifies only naturally occurring target coding sequence, or related sequences. Probes for the detection of related nucleic acid sequences are selected from conserved or highly conserved nucleotide regions of target family members and such probes may be used in a pool of degenerate probes. For the detection of identical nucleic acid sequences, or where maximum specificity is desired, nucleic acid probes are selected from the non-conserved nucleotide regions or unique regions of the target polynucleotides. As used herein, the term "non-conserved nucleotide region" refers

to a nucleotide region that is unique to a target coding sequence disclosed herein and does not occur in related family members.

PCR as described in US-A-4683195, US-A-4800195 and US-A-4965188 provides  
5 additional uses for oligonucleotides based upon target sequences. Such oligomers are generally chemically synthesized, but they may be generated enzymatically or produced from a recombinant source. Oligomers generally comprise two nucleotide sequences, one with sense orientation (5'→3') and one with antisense (3'←5') employed under optimised conditions for identification of a specific gene or condition.

10 The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantification of closely related DNA or RNA sequences.

The nucleic acid sequence for a target can also be used to generate hybridisation  
15 probes as previously described, for mapping the endogenous genomic sequence. The sequence may be mapped to a particular chromosome or to a specific region of the chromosome using well known techniques. These include *in situ* hybridisation to chromosomal spreads (Verma *et al* (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York City), flow-sorted chromosomal  
20 preparations, or artificial chromosome constructions such as YACs, bacterial artificial chromosomes (BACs), bacterial PI constructions or single chromosome cDNA libraries.

*In situ* hybridisation of chromosomal preparations and physical mapping techniques  
5 such as linkage analysis using established chromosomal markers are invaluable in extending genetic maps. Examples of genetic maps can be found in Science (1995; 270:410f and 1994; 265:1981f). Often the placement of a gene on the chromosome of another mammalian species may reveal associated markers even if the number or arm of a particular human chromosome is not known. New sequences can be  
30 assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once a disease or syndrome has been crudely localised by genetic linkage to a particular genomic region any sequences mapping to that area may represent associated or regulatory genes for further  
35 investigation. The nucleotide sequence of the subject invention may also be used to detect differences in the chromosomal location due to translocation, inversion, etc. between normal, carrier or affected individuals.



## ORGANISM

The term "organism" in relation to the present invention includes any organism that  
5 could comprise the target and/or products obtained therefrom. Examples of organisms  
may include a fungus, yeast or a plant.

The term "transgenic organism" in relation to the present invention includes any  
organism that comprises the target and/or products obtained.

10

## TRANSFORMATION OF HOST CELLS/HOST ORGANISMS

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism.  
Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Teachings  
15 on the transformation of prokaryotic hosts is well documented in the art, for example  
see Sambrook et al (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold  
Spring Harbor Laboratory Press) and Ausubel *et al.*, Current Protocols in Molecular  
Biology (1995), John Wiley & Sons, Inc.

20 If a prokaryotic host is used then the nucleotide sequence may need to be suitably  
modified before transformation - such as by removal of introns.

In another embodiment the transgenic organism can be a yeast. In this regard, yeast  
have also been widely used as a vehicle for heterologous gene expression. The  
25 species *Saccharomyces cerevisiae* has a long history of industrial use, including its use  
for heterologous gene expression. Expression of heterologous genes in  
*Saccharomyces cerevisiae* has been reviewed by Goodey et al (1987, Yeast  
Biotechnology, D R Berry et al, eds, pp 401-429, Allen and Unwin, London) and by King  
et al (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds,  
30 pp 107-133, Blackie, Glasgow).

For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene  
expression. First, it is non-pathogenic to humans and it is incapable of producing  
certain endotoxins. Second, it has a long history of safe use following centuries of  
35 commercial exploitation for various purposes. This has led to wide public acceptability.  
Third, the extensive commercial use and research devoted to the organism has resulted

in a wealth of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting the nucleotide sequence of the present invention into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the nucleotide sequence of the present invention, usually a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

For the transformation of yeast several transformation protocols have been developed. For example, a transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen et al (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito, H et al (1983, J Bacteriology 153, 163-168).

The transformed yeast cells are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, eg G418.

Another host organism is a plant. The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material. Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector

system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

5

Thus, the present invention also provides a method of transforming a host cell with a nucleotide sequence that is to be the target or is to express the target. Host cells transformed with the nucleotide sequence may be cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture. The protein  
10 produced by a recombinant cell may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing coding sequences can be designed with signal sequences which direct secretion of the coding sequences through a particular prokaryotic or eukaryotic cell membrane. Other recombinant constructions  
15 may join the coding sequence to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins (Kroll *DJ et al* (1993) DNA Cell Biol 12:441-53).

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15 According to one aspect of the present invention, the additional target is a P<sub>cAMP</sub> target, which P<sub>cAMP</sub> target is PDE (phosphodiesterase), in particular a PDE which is a cAMP hydrolysing PDE (and optionally cGMP hydrolysing).

It is known that cyclic nucleotides, such as cAMP and cGMP, are important intracellular second messengers. Cyclic nucleotide phosphodiesterases - otherwise known as PDEs - are a family of enzymes that catalyse the degradation of cyclic nucleotides and, in doing so, are one of the cellular components that regulate the concentration of cyclic nucleotides.

5 In recent years, at least seven PDE enzymes (such as PDEI - PDEVII), as well as many subtypes of these enzymes, have been defined based on substrate affinity and cofactor requirements (Beavo JA and Reifsnyder DH, Trends Pharmacol. Sci. 11:150 [1990]; Beavo J, In: Cyclic Nucleotide Phosphodiesterases: Structure, Regulation and Drug Action., Beavo J and Housley MD (Eds.). Wiley:Chichester, pp. 3-15 [1990]).

30 Examples of PDEs include: PDEI which is a Ca<sup>2+</sup>/Calmodulin-dependent PDE; PDEII which is a cAMP and cGMP stimulated PDE; PDEIII which is a cGMP inhibited PDE; PDEIV which is a high affinity cAMP-specific PDE; and PDEV which is a cGMP specific PDE. PDEI etc. are sometimes called PDE type I etc. or PDE type 1 etc.

35 Each PDE family may contain two or more isoforms (i.e. there may be two or more PDE isoenzymes). By way of example, mammalian PDE IV, the homologue of the

*Drosophila* Dunce gene (Chen CN *et al.*, Proc. Nat. Acad. Sci. (USA) 83:9313 [1986]), is known to have four isoforms in the rat (Swinnen JV *et al.*, Proc. Nat. Acad. Sci. (USA) 86:5325 [1989]). Human PDEs are also known to occur as isoforms and have splice variants. For example, the cloning of one human isoform of PDEIV from monocytes was reported in 1990 (Livi GP *et al.*, Mol. Cell. Bio., 10:2678 [1990]). By way of further example, other workers have independently cloned three splice variants of PDEIV, which are now designated hPDEIV-B1, hPDEIV-B2, and hPDEIV-B3.

Teachings on cyclic nucleotide phosphodiesterases can also be found in US-A-5932423 and US-A-5932465.

Teachings on a further cyclic nucleotide phosphodiesterase - namely CN PCDE8 - can be found in WO-A-97/35989. According to WO-A-97/35989, CN PCDE8 has two isozymes - which were designated CN PCDE8A and CN PCDE8B. The term "isozyme" is sometimes referred to in the art as "isoform".

According to WO-A-97/35989, many inhibitors of different PDEs have been identified and some have undergone clinical evaluation. For example, PDEIII inhibitors are being developed as antithrombotic agents, as antihypertensive agents and as cardiotonic agents useful in the treatment of congestive heart failure. Rolipram, a PDEIII inhibitor, has been used in the treatment of depression and other inhibitors of PDEIII are undergoing evaluation as anti-inflammatory agents. Rolipram has also been shown to inhibit lipopolysaccharide (LPS) induced TNF-alpha which has been shown to enhance HIV-1 replication *in vitro*. Therefore, rolipram may inhibit HIV-1 replication (Angel *et al* 1995 AIDS 9:1137-44). Additionally, based on its ability to suppress the production of TNF alpha and beta and interferon gamma, rolipram has been shown to be effective in the treatment of encephalomyelitis, the experimental animal model for multiple sclerosis (Sommer *et al*, 1995 Nat Med 1:244-248) and may be effective in the treatment of tardive dyskinesia (Sasaki *et al*, 1995 Eur J Pharmacol 282:71-76).

According to WO-A-97/35989, there are also non-specific PDE inhibitors such as theophylline, used in the treatment of bronchial asthma and other respiratory diseases, and pentoxifylline, used in the treatment of intermittent claudication and diabetes-induced peripheral vascular disease. Theophylline is thought to act on airway smooth muscle function as well as in an anti-inflammatory or immunomodulatory capacity in the treatment of respiratory diseases (Banner *et al*

1995 Respir J 8:996-1000) where it is thought to act by inhibiting both CN PDE cAMP and cGMP hydrolysis (Banner *et al* 1995 Monaldi Arch Chest Dis 50:286-292). Pentoxifylline, also known to block TNF-alpha production, may inhibit HIV-1 replication (Angel *et al supra*). A list of CN PDE inhibitors is given in Beavo 1995 *supra*.

It has been suggested that selective inhibitors of PDEs, in addition to their isozymes and their subtypes, will lead to more effective therapy with fewer side effects. For example, see the teachings in the reviews of Wieshaar RE *et al*, (J. Med. Chem., 28:537 [1985]), Giembycz MA (Biochem. Pharm., 43:2041 [1992]) and Lowe JA and Cheng JB (Drugs of the Future, 17:799-807 [1992]).

Thus, for some applications it is desirable to have a selective inhibition of an individual type of PDE.

Background teachings on PDEs have been presented by Victor A. McKusick *et al* on <http://www3.ncbi.nlm.nih.gov/Omim/searchomim.htm>. The following information concerning PDE2 or cGMP-stimulated PDE, has been extracted from that source.

"Cyclic nucleotides serve as second messengers that mediate a variety of cellular responses to extracellular signals such as hormones, light, and neurotransmitters. Cyclic nucleotide phosphodiesterases (PDEs) play a role in signal transduction by regulating the cellular concentrations of cyclic nucleotides. Mammalian cells contain multiple PDEs that are distinguished into at least 7 families based on their substrate affinity and on their selective sensitivity to cofactors and inhibitory drugs. These families are: (I) Ca(2+)/calmodulin-dependent PDEs; (II) cGMP-stimulated PDEs; (III) cGMP-inhibited PDEs; (IV) cAMP-specific PDEs; (V) cGMP-specific PDEs; (VI) photoreceptor PDEs; and (VII) high-affinity, cAMP-specific. From the amino acid sequences, it is evident that all these PDE families contain a related domain, thought to be the catalytic domain, with approximately 30% sequence identity between families. Members of the same family are more closely related; they share 60 to 80% sequence identity throughout the entire coding region.

Michaeli *et al*. (1993) established a highly sensitive functional screen for the isolation of cDNAs encoding cAMP phosphodiesterases by complementation of defects in the *Saccharomyces cerevisiae* strain lacking both endogenous cAMP PDE genes, PDE1 and PDE2. Three groups of cDNAs corresponding to 3 distinct human genes encoding cAMP-specific PDEs were isolated from a human glioblastoma cDNA library using this functional screen. Two of the genes were closely related to the *Drosophila*

'dunce' cAMP-specific PDE. The third gene, which Michaeli et al. (1993) referred to as HCP1, encoded a novel cAMP-specific PDE. HCP1 had an amino acid sequence related to the sequences of the catalytic domains of all cyclic nucleotide PDEs. It is a high-affinity cAMP-specific PDE that does not share other properties of the cAMP-specific PDE family, however. The PDE activity of HCP1 was not sensitive to cGMP or other inhibitors of the cGMP-inhibitable PDEs. The biochemical and pharmacologic properties of HCP1 suggested to Michaeli et al. (1993) that it is a member of a previously undiscovered cyclic nucleotide PDE family, which they designated as family VII. Northern blot analysis indicated the presence of high levels of an HCP1 RNA in human skeletal muscle.

By Southern blot analysis of somatic cell hybrid lines, Milatovich et al. (1994) mapped the HCP1 locus to chromosome 8; by study of somatic cell hybrid lines that contained different regions of chromosome 8, they regionalized the assignment to 8q13-q22. Han et al. (1998) mapped the PDE7A gene to 8q13 by fluorescence in situ hybridization. By interspecific backcross analysis, they mapped the mouse Pde7A gene to the proximal region of chromosome 3."

Background teachings on PDE2 have been presented by Jennifer P. Macke et al on <http://www3.ncbi.nlm.nih.gov/Omim/searchomim.htm>. The following information concerning PDE2 cGMP-stimulated has been extracted from that source.

"Rosman et al. (1997) cloned a cDNA corresponding to human PDE2A. The PDE2A gene encodes a 941 amino acid polypeptide with a predicted molecular mass of 106 kD. The protein sequence is 90% identical to bovine and rat PDE2A sequences. Northern blot analysis showed that PDE2A was expressed as a 4.2-kb mRNA at varying levels in all human tissues tested, with greatest expression in brain. Expression studies revealed that PDE2A hydrolyzes cAMP and cGMP and is inhibited by the PDE2A-specific inhibitor EHNA."

Nucleotide sequences and amino acid sequences for PDEs are available in the literature. Some sequences are presented in the Sequence Listings provided herein.

In one aspect, the PDE target is selected from any one or more of the following PDE enzymes: PDE<sub>CAMP</sub> 1, PDE<sub>CAMP</sub> 2, PDE<sub>CAMP</sub> 3, PDE<sub>CAMP</sub> 4, PDE5, PDE<sub>CAMP</sub> 7 and PDE<sub>CAMP</sub> 8.

In a more preferred aspect, the PDE target is selected from any one or more of the following PDE enzymes: PDE<sub>CAMP</sub> 1, PDE<sub>CAMP</sub> 2, PDE<sub>CAMP</sub> 3, PDE<sub>CAMP</sub> 4 and PDE%.



Preferably, for the present invention, the PDE to target is at least PDE 5.

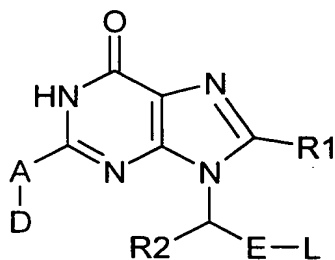
### I:PDE

- 5 As indicated above, the additional agent may be any suitable agent that can act as an I:PDE. In addition, or in the alternative, the agent of the present invention may also act as an I:PDE.

10 Examples of I:PDE are disclosed in EP-A-091133, EP-A-0771799 and in EP-A-0771799.

For convenience, claim 1 of EP-A-0771799 is now repeated:

A purin-6-one derivative with general formula (I):



15

wherein:

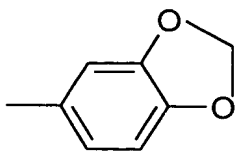
- |                                   |  |
|-----------------------------------|--|
| R <sup>1</sup>                    | represents hydrogen or a linear or branched alkyl containing up to 8 carbon atoms;   |
| R <sup>2</sup>                    | represents a linear or branched acyl containing up to 4 carbon atoms, or a linear or branched alkyl containing up to 8 carbon atoms optionally substituted by hydroxyl, azido or a group with formula -NR <sup>3</sup> R <sup>4</sup> or -OSO <sub>2</sub> R <sup>5</sup> ; wherein  |
| R <sup>3</sup> and R <sup>4</sup> | are identical or different and represent a cycloalkyl containing 3 to 6 carbon atoms, hydrogen, formyl, or a linear or branched alkyl containing up to 6 carbon atoms, optionally substituted by a linear or branched alkoxy or alkoxy carbonyl respectively containing up to 6 carbon atoms or by a group with formula -(CO) <sub>a</sub> -NR <sup>6</sup> R <sup>7</sup> , wherein |

- a is the number 0 or 1;
- $R^6$  and  $R^7$  are identical or different and represent hydrogen, formyl, hydroxyl, phenyl or a linear or branched alkyl containing up to 6 carbon atoms, optionally substituted by hydroxyl or a linear or branched alkoxy containing up to 5 carbon atoms; or
- $R^3$  and/or  $R^4$  represent a linear or branched alkoxy carbonyl containing up to 6 carbon atoms, carboxyl or a linear or branched acyl containing up to 6 carbon atoms optionally substituted by hydroxyl or a linear or branched alkoxy containing up to 4 carbon atoms; or
- $R^3$  and/or  $R^4$  represent a residue with formula  $-(CO)_b-T-NR^8R^9$ ,  $-CO-R^{10}$ ,  $-SO_2R^{11}$  or  $-SO_2NR^{12}R^{13}$ , wherein
- b has the meaning given above for a and is identical thereto or different therefrom;
- T can represent a linear or branched alkyl containing up to 5 carbon atoms, or when  $b \neq 0$  it can also represent a bond;
- $R^8$  and  $R^9$  have the meaning given for  $R^6$  and  $R^7$  above and are identical thereto or different therefrom;
- $R^{10}$  represents a saturated, partially unsaturated or unsaturated 5- to 7-membered heterocycle containing up to 3 heteroatoms selected from S, N and/or O, which can optionally also be substituted on the N function by a linear or branched alkyl, alkoxy or alkoxy carbonyl containing up to 4 carbon atoms, carboxyl, benzyloxycarbonyl or hydroxyl;
- $R^{11}$  represents a linear or branched alkyl containing up to 5 carbon atoms, benzyl or phenyl;
- $R^{12}$  and  $R^{13}$  are identical or different and represent hydrogen, phenyl or a linear or branched alkyl containing up to 6 carbon atoms; or

- $R^3$  and  $R^4$  together with the nitrogen atom form a 5- or 6-membered saturated, partially unsaturated or unsaturated heterocycle which can contain up to 3 heteroatoms selected from N, S and/or O or a  $-NR^{14}$  residue, and which is optionally substituted by carbonyl, a linear or branched alkoxycarbonyl containing up to 5 carbon atoms or a linear or branched alkyl containing up to 5 carbon atoms which in its turn can be substituted by hydroxyl, carboxy or a linear or branched acyl, alkoxy or alkoxycarbonyl respectively, containing up to 6 carbon atoms; wherein
- $R^{14}$  represents hydrogen, carbonyl or a linear or branched alkyl or alkoxycarbonyl respectively containing up to 5 carbon atoms; and
- $R^5$  represents phenyl or a linear or branched alkyl containing up to 5 carbon atoms;
- A represents a linear or branched alkylene or alkenylene chain respectively containing up to 6 carbon atoms;
- D and L are identical or different and represent an aryl containing 6 to 10 carbon atoms or a 5- to 7-membered aromatic, optionally benzocondensed heterocycle containing up to 3 heteroatoms selected from S, N and/or O, optionally substituted up to 3 times, identically or differently, by a halogen, hydroxyl, nitro, trifluoromethyl, carboxy, a linear or branched alkyl, alkoxy or alkoxycarbonyl respectively containing up to 6 carbon atoms or by a group with formula  $-(V)_c-NR^{15}R^{16}$  and/or  $-OR^{17}$ ; wherein
- c is the number 0 or 1;
- V represents a residue with formula  $-CO$  or  $-SO_2$ ;
- $R^{15}$  and  $R^{16}$  are identical or different and have the meaning given for  $R^3$  and  $R^4$  above;

- $R^{17}$  represents hydrogen, a linear or branched alkenyl containing up to 8 carbon atoms or a linear or branched alkyl containing up to 8 carbon atoms, optionally substituted up to 3 times, identically or differently, with hydroxyl, carbonyl or linear or branched alkoxy carbonyl containing up to 5 carbon atoms; and/or the cycles are optionally substituted by an aryl containing 6 to 10 carbon atoms or by a 5- to 7-membered aromatic, optionally benzocondensed heterocycle containing up to 3 heteroatoms selected from S, N and/or O, which in its turn is optionally substituted up to two times, identically or differently, by a halogen, hydroxyl, nitro, carboxyl, trifluoromethyl or a linear or branched alkyl, alkoxy or alkoxy carbonyl respectively containing up to 5 carbon atoms or with a group with formula  $(V')_d-NR^{18}R^{19}$ ; wherein
- $d$  has the meaning given above for  $a$  and is identical thereto or different therefrom;
- $R^{18}$  and  $R^{19}$  have the meaning given above for  $R^3$  and  $R^4$  and are identical thereto or different therefrom;
- $V'$  has the meaning given above for  $V$  and is identical thereto or different therefrom; and/or
- represents the ring system given below for  $D$ , optionally substituted by a linear or branched acyl containing up to 5 carbon atoms, optionally substituted by hydroxyl, a linear or branched alkoxy containing up to 5 carbon atoms or by a group with formula  $-NR^{20}R^{21}$ ; wherein
- $R^{20}$  and  $R^{21}$  are identical or different and have the meaning given above for  $R^3$  and  $R^4$ ; or
- $E$  Represents a residue with formula  $-CH_2-Y-Z-$ ; wherein
- $Y$  Represents a bond or an oxygen or sulphur atom or the group  $-NH-$ ;
- $Z$  Represents a linear or branched alkyl chain containing up to 5 carbon atoms;

D represents a residue with formula



5 and tautomers and salts thereof.

Further I:PDEs can be selected from the following structures:

<u>Compound</u>	<u>Structure</u>	<u>Mode of action</u> <u>References</u>
<u>Fla</u>		I:PDE1 EP-A-0911333 (Example 50)
<u>Fib</u>		I:PDE2 EHNA (also an inhibitor of Adenosinedeaminase)
FII		I:PDE2 EP-A-0771799 (Example 100)
FIII		I:PDE3 Milrinone (which is commercially available)
FIV		I:PDE4 Rolipram (which is commercially available)

VIP (vasoactive intestinal peptide)

5 According to one aspect of the present invention, an additional target is a  $P_{\text{cAMP}}$  target, which  $P_{\text{cAMP}}$  target is VIP or one of its associated receptors. Current classification/nomenclature refers to these as VPAC1, VPAC2 and PACAP.

10 Nucleotide sequences and amino acid sequences for VIP and its receptors are available in the literature. Some sequences are presented in the Sequence Listings provided herein.

VIP is a major endogenous neurotransmitter released during sexual arousal. The vasodilatory effects of VIP are mediated by adenylate cyclase activation and cAMP  
15 production. Without wishing to be bound by theory, this effect may be mediated via VIP receptor subtypes VPAC<sub>1</sub>, VPAC<sub>2</sub> or PACAP (pituitary adenylate cyclase-activating peptide) receptors. VPAC<sub>2</sub> and PACAP receptors are most widely expressed in the CNS and the receptors despite being expressed in the pituitary, appears to have no widespread biological function.

20

The agent could potentiate VIP and/or act as a VIP mimetic or analogue thereof. The agent would then potentiate and/or mimic the vasorelaxant effects of endogenous VIP released during sexual arousal. The agent may also have an additive effect on VIP-induced relaxations of male genital smooth muscle.

5

Background teachings on VIP and its associated receptors are presented by Victor A. McKusick et al on <http://www3.ncbi.nlm.nih.gov/Omim/searchomim.htm>. The following text concerning VIP has been extracted from that source.

30 "Vasoactive intestinal peptide (VIP), a 28-amino acid peptide originally isolated from porcine duodenum, is present not only in gastrointestinal tissues but also in neural tissues, possibly as a neurotransmitter, and exhibits a wide variety of biological actions. Because VIP shows similarities to glucagon, secretin and gastric inhibitory peptide (GIP), it has been considered a member of the glucagon-secretin family. The  
35 primary translation product of the mRNA encoding VIP (prepro-VIP) has a molecular weight of 20 daltons. By cloning the DNA sequence complementary to the mRNA coding for human VIP, Itoh et al. (1983) found that the VIP precursor contains not

only VIP but also a novel peptide of 27 amino acids, designated PHM27, that has aminoterminal histidine and carboxyterminal methionine. It differs from PHI17 isolated from porcine intestine by 2 amino acids; PHI27, as its designation indicates, has carboxyterminal isoleucine. Linder et al. (1987) isolated the human gene for VIP and PHM27 and studied its expression in various tissues of the rat. Heinz-Erian et al. (1985) suggested that deficient innervation of sweat glands of cystic fibrosis patients by the VIP neuropeptide might be a basic mechanism for the decreased water content and relative impermeability of the epithelium to chloride and other ions that characterize cystic fibrosis. To test this hypothesis, Gozes et al. (1987) took the 'candidate gene' approach. Bodner et al. (1985) had shown that VIP and PHM-27 are encoded by adjacent exons. Gozes et al. (1987) used the PHM-27-encoding genomic fragment to detect the presence of the VIP gene at 6q21-qter. Thus, they eliminated a defective VIP gene as a candidate for the primary cause of cystic fibrosis (which is coded by chromosome 7). By in situ hybridization techniques, Gozes et al. (1987) assigned the VIP gene to 6q24. This placed VIP in the region of MYB (189990), which has been mapped to 6q22. Gozes et al. (1987) investigated a functional relationship between the 2 genes in neuronal tissue. They observed a sharp peak of MYB mRNA in the hippocampus of 3-day-old rats, preceding the peak of VIP mRNA that occurs in this area at 8 days of age. Omary and Kagnoff (1987) found nuclear receptors for VIP in a human colonic adenocarcinoma cell line. Gotoh et al. (1988) assigned VIP to chromosome 6 by spot blot hybridization of a molecularly cloned fragment of the gene to sorted chromosomes. The localization was refined to 6q26-q27 by in situ hybridization."

As indicated, background teachings on VIP and its associated receptors are presented by Victor A. McKusick et al (ibid). The following text concerning VIPR1 or VPAC1 has been extracted from that source.

5        "Vasoactive intestinal peptide (VIP; 192320) is an octacosameric neuroendocrine  
mediator found predominantly in cholinergic presynaptic neurons of the central  
nervous system and in peripheral peptidergic neurons innervating diverse tissues. Of  
the many neuroendocrine peptides with immunologic functions, VIP is distinguished  
10        by its capacity to affect both B and T cells directly. Distinct subsets of neural,  
respiratory, gastrointestinal, and immune cells bear specific high-affinity receptors for  
VIP, which are associated with a guanine nucleotide-binding (G) protein capable of  
activating adenylate cyclase. Libert et al. (1991) obtained 4 new receptors of the G  
protein-coupled receptor family by selective amplification and cloning from thyroid  
cDNA. One of these, termed RDC1, was identified as the VIP receptor by  
15        Sreedharan et al. (1991). Libert et al. (1991) mapped the VIPR gene to 2q37 by in  
situ hybridization. Later information made it doubtful that the gene mapped to 2q37  
was in fact the VIP receptor gene (Vassart, 1992). The sequence that was  
designated GPRN1 by Sreedharan et al. (1991) and mapped to 2q37 was found not  
to bind VIP by Wenger (1993). Sreedharan et al. (1995) isolated an authentic type I  
20        VIP receptor gene and by fluorescence in situ hybridization localized it to the 3p22  
band in a region associated with small-cell lung cancer. By interspecific backcross  
analysis, Hashimoto et al. (1999) mapped the mouse *Vipr1* gene to the distal region  
of chromosome 9, a region that shows homology of synteny with human chromosome  
3p. Sreedharan et al. (1993) cloned a human intestinal VIP receptor gene; the  
25        deduced amino acid sequence shares 84% identity with the rat lung VIP receptor.  
Couvineau et al. (1994) isolated 2 VIPR cDNA clones from a human jejunal epithelial  
cell cDNA library. One encodes a VIP receptor consisting of 460 amino acids and  
having 7 putative transmembrane domains, as do other G protein-coupled receptors.  
The other encodes a 495-amino acid VIP receptor-related protein exhibiting 100%  
30        homology with the functional VIP receptor over the 428 amino acids at the C-terminal  
region, but containing a completely divergent 67-amino acid N-terminal domain.  
When expressed in COS-7 cells, the second protein did not bind radioiodinated VIP,  
although it was normally addressed at the plasma membrane as assessed by  
immunofluorescence studies. The type I VIP receptor, also termed type II PACAP  
35        receptor (see 102981 for another type of PACAP receptor), was found by Sreedharan  
et al. (1995) to span approximately 22 kb and to be comprised of 13 exons (ranging  
from 42 to 1,400 bp) and 12 introns (ranging from 0.3 to 6.1 kb). Sreedharan et al.  
(1995) also characterized the promoter and the 5-prime flanking region of the gene."



As indicated, background teachings on VIP and its associated receptors are presented by Victor A. McKusick et al (ibid). The following text concerning VIPR2 or VPAC2 has been extracted from that source.

5       “Vasoactive intestinal peptide (VIP; 192320) and pituitary adenylate cyclase activating polypeptide (PACAP; 102980) are homologous peptides that function as neurotransmitters and neuroendocrine hormones. While the receptors for VIP and PACAP share homology, they differ in their substrate specificities and expression patterns. See VIPR1 (192321) and ADCYAP1R1(102981). Svoboda et al. (1994) performed low stringency PCR using primers based on sequences conserved among  
10       VIP receptors. They cloned the human VIP2 receptor gene from a lymphoblast cDNA library. This gene encoded a 438-amino acid polypeptide that shares 86% identity with the rat VIP2 receptor. They expressed the human VIP2 receptor in Chinese hamster ovary cells and found that it binds to PACAP-38, PACAP-27, VIP, and helodermin, and that binding of the receptor to any of these peptides activates  
15       adenylate cyclase. Peptide binding was found to be inhibited by GTP. Adamou et al. (1995) cloned the VIP2 receptor gene from a human placenta cDNA library. Northern blotting revealed that VIPR2 is expressed as 2 transcripts of 4.6 kb and 2.3 kb at high levels in skeletal muscle and at lower levels in heart, brain, placenta, and pancreas.  
20       Mackay et al. (1996) used fluorescence in situ hybridization to map the VIPR2 gene to human chromosome 7q36.3. Further mapping with cell lines derived from patients with holoprosencephaly type 3 (HPE3; 142945) revealed that the VIPR2 gene lies within the HPE3 minimal critical region. Mackay et al. (1996) stated that although VIPR2 may contribute to the HPE3 phenotype, it is not the sole factor responsible.”

#### 25       AC (adenylate cyclase)

According to one aspect of the present invention, an additional target is a  $P_{cAMP}$  target, which  $P_{cAMP}$  target is AC.

30       Nucleotide sequences and amino acid sequences for AC are available in the literature.

Background teachings on AC are presented by Victor A. McKusick et al on <http://www3.ncbi.nlm.nih.gov/Omim/searchomim.htm>. The following text concerning  
35       AC has been extracted from that source.

“Adenylyl cyclase (EC 4.6.1.1 ) catalyzes the transformation of ATP into cyclic AMP. The enzymatic activity is under the control of several hormones, and different

polypeptides participate in the transduction of the signal from the receptor to the catalytic moiety. Stimulatory or inhibitory receptors (Rs and Ri) interact with G proteins (Gs and Gi) that exhibit GTPase activity and they modulate the activity of the catalytic subunit of the adenylyl cyclase. Parma et al. (1991) cloned a cDNA  
5 corresponding to human brain adenylyl cyclase, symbolized by them as HBAC1. By in situ hybridization to metaphase chromosomal spreads using the human brain cDNA probe, Stengel et al. (1992) showed that the gene is located on 8q24.2. A highly homologous gene, ADCY2 (103071), was assigned to 5p15.3 by the same method."

## GENERAL RECOMBINANT DNA METHODOLOGY TECHNIQUES

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A  
15 Laboratory Manual (1989) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4<sup>th</sup> Ed, John Wiley & Sons, Inc. PCR is described in US-A-4683195, US-A-4800195 and US-A-4965188.

## SUMMARY

20 In summation, the present invention relates to the use of an I:NPY to treat MED, and in particular use of a selective NPY Y1 receptor antagonist.

A further preferred object of the present invention relates to the use of a selective  
25 NPY Y1 receptor antagonist in combination with a potent and selective cGMP PDE5 inhibitor for the treatment of MED.

## TEST METHODS

## ANIMAL TEST MODELS

*In vivo* models may be used to investigate and/or design therapies or therapeutic  
35 agents to treat MED. The models could be used to investigate the effect of various tools/lead compounds on a variety of parameters which indicate the sexual arousal

response. These animal test models can be used as, or in, the assay of the present invention. The animal test model will be a non-human animal test model.

5 There are a number of animal models for male sexual dysfunction (MSD) available that could be used.

By way of example, reference may be made to invasive animal models, or to non-invasive animal models.

10

### AN ASSAY FOR MEASURING cAMP ACTIVITY/LEVELS

15 Measurement of cAMP from corpus cavernosal tissue samples using a Biotrak cAMP Enzymeimmunoassay (EIA) kit (Amersham Life Sciences RPN 225).

cAMP levels are measured by EIA in corpus cavernosal tissue samples. The EIA is based on competition between unlabelled cAMP and a fixed quantity of peroxidase labelled cAMP for a limited amount of cAMP specific antibody.

20

#### 1. MATERIALS

All materials are supplied by Amersham Life Science cAMP EIA kit (RPN 225) unless otherwise stated.

5

1.1 Microtitre plate - 96 well plate coated with donkey anti-rabbit IgG.

1.2 Assay buffer - 0.05M sodium acetate buffer pH5.8 containing 0.02% bovine serum albumin and 0.5% preservative upon reconstitution. The contents of the bottle are transferred to a graduated cylinder using 3x15ml distilled water washes. The  
30 final volume is then adjusted to 500ml.

1.3 cAMP standard (for acetylation method). cAMP at 10.24pmol/ml in 0.05M acetate buffer pH5.8 containing 0.02% bovine serum albumin and 0.5% preservative upon reconstitution. Standard is dissolved in 2.5ml of assay buffer for use.

1.4 Antiserum. Anti-cAMP antibody in 0.05M acetate buffer pH5.8 containing 0.02%  
35 bovine serum albumin and 0.5% preservative upon reconstitution. Prior to use,

antibody is diluted with 11ml assay buffer and mixed by gentle inversion to dissolve contents.

1.5 cAMP conjugate. cAMP horseradish peroxidase in 0.05M acetate buffer pH5.8 containing 0.02% bovine serum albumin and 0.5% preservative upon reconstitution.

5 Prior to use, solution is diluted with 11ml assay buffer and mixed by gentle inversion to dissolve contents.

1.6 Wash buffer. 0.01M phosphate buffer pH7.5 containing 0.05% (v/v) Tween™ 20 upon reconstitution. The contents of the bottle are transferred to a graduated cylinder using 3x15ml distilled water washes. The final volume is then adjusted to

10 500ml.

1.7 TMB substrate. 3,3', 5,5'- tetramethylbenzidine (TMB)/ hydrogen peroxide, in 20% (v/v) dimethylformamide.

Ready for use.

1.8 Acetylation reagent. 2ml acetic anhydride, 4ml triethylamine, prepared as

15 required.

1.9 Sulphuric acid (1M). 1M Sulphuric acid is prepared from an 18M stock (BDH).

1.11ml of acid is added to 18.8ml of distilled water.

## 2. SPECIFIC EQUIPMENT

20

2.1 Disposable 5ml glass test tubes

2.2 Spectrophotometric plate reader (Spectra max 190)

2.3 Microtitre plate shaker (Luckham R100)

## 3. METHODS

25

• Tissue sample preparation. The tissues were treated with the relevant pretreatment in 5ml samples of physiological salt solution eg agonists, cAMPmimetics etc. After treatment the samples were snap frozen in liquid nitrogen

30

and then smashed using a hammer. The powder was scraped into a centrifuge tube and 1ml of 0.5M ice cold perchloric acid (PCA) was added. The sample was vortex mixed and left on ice for 1hr.

• cAMP extraction from tissue samples. The samples were centrifuged at 10000g for 5 min at 4°C. The supernatant was removed and placed in other centrifuge tubes.

35

The pellet was kept for protein analysis at -80°C. The supernatant samples were then neutralised to pH~6 using K<sub>3</sub>PO<sub>4</sub>. Centrifuged at 10000g for 5 min at 4°C.

Recover supernatant and wash 4 times with 5 volumes (5ml) of water saturated diethyl ether. The upper ether layer should be discarded after each wash. Transfer aqueous to into a short thin glass tube and dry under a stream of nitrogen at 60°C. Dissolve dried extract in 1ml of assay buffer and store in refrigerator until required (or  
5 can be frozen).

- Stock reagents are equilibrated to room temperature and working solutions then prepared

- cAMP standards are prepared in glass tubes labelled 2, 4, 8, 16, 32, 64, 128, 256, and 512fmol. This is achieved by adding 1ml of assay buffer to all tubes except  
10 the 512fmol standard. 1ml of acetylation standard (10.24pmol/ml) is then added to the two top standards (256, and 512fmol). The 256fmol standard is vortexed and 1ml transferred to the 128fmol standard. This is continued until the 2fmol standard where 1ml of solution is disposed of. A zero standard tube is set up containing 1ml of assay buffer.

- 15 • Tissue extract samples are thawed on ice (if necessary) and diluted 1 in 100 (10µl sample to 990µl assay buffer) in labelled glass tubes.

- The cAMP in all standards and samples is acetylated by the addition of 100µl of acetylation reagent in a fume hood which is added down the side of the tube before immediately vortexing.

- 20 • 50µl of all standards and samples are added to the appropriate wells of the 96 well plate, and 150µl of assay buffer is added to non specific binding (NSB) wells.

- 100µl of antiserum is added to all wells except blanks (B) and NSB before incubating for 2 hours at 3-5°C.

- After incubation, 100µl of cAMP-peroxidase conjugate is added to all wells except  
25 B before a further 1 hour incubation at 3-5°C.

- Plates are emptied by turning them upside down and blotting onto absorbent paper before washing each well four times with 400µl of wash buffer. After each wash plates are re-blotted to ensure any residual wash buffer is removed. 200µl TMB is then immediately dispensed into all wells.

- 30 • Plates are put on a plate shaker for 30 minutes at room temperature before the addition of 100µl of 1M sulphuric acid into all wells. The optical density is read on Spectra max 190 at 450nm within 30 minutes.

#### 4. STANDARDS

35

With each assay the following standard tubes are set up:-

#### 4.1 Spiking a standard in assay buffer

A known amount of cAMP is spiked into assay buffer to determine the efficiency of the assay. 70pmol/ml of cAMP is added to assay buffer which is equivalent to 35fmol/well in the assay, which is in the middle of the dose response curve.

To make up 1ml of standard:- 68.4µl 521fmol/well standard  
931.6µl Assay buffer

#### 4. Effects of compounds on plate

Standards are set up to determine whether the compound used in the functional studies has any effect on the 96 well plate or affects the binding of cAMP.

These include:-

- Spiking the compound into assay buffer alone to assess the effects of the compound directly on the plate.
- Spiking the compound into plasma containing basal levels of cAMP to assess the effects of the compound on the binding of cAMP to the plate.

5nM concentrations of compound are spiked into each standard. 5nM is chosen because total drug levels at the end of infusion have in the past been approximately 150-300nM. Samples are diluted 1:100 before being assayed, therefore 5nM allows for any larger than expected total drug concentrations at the end of infusion.

#### 5. CALCULATIONS

The Spectra max plate reader reads the optical density (OD) at 450nm.

The standard curve is generated by plotting the %B/Bo (y axis) against cAMP fmol/well (x axis) on Spectra max.

%B/Bo (% bound) for each sample and standard is calculated as follows:-

Bo = zero standard (see methods 3.2)

$\%B/Bo = \frac{(\text{standard or sample OD} - \text{NSB OD})}{\text{Bo OD}} \times 100$

(Bo OD-NSB OD)

The fmol/well volume can then be read directly from the standard curve for each sample. Values are then converted to pmol/ml before taking the mean of each pair of samples.

#### Conversion of values from fmol/well to pmol/ml:-

fmol to pmol = divide by 1000

Volume in well = 50µl .... So (x1000)  
50

Sample is diluted 1/100, so overall =  $1 \times 1000/1000 \times 100/50 = 2$

So all fmol/well values are multiplied by 2 to give pmol/ml

#### ANIMAL TEST MODEL

POTENTIATING THE EFFECTS OF CYCLIC ADENOSINE-3',5'-  
MONOPHOSPHATE (cAMP) RESULTS IN INCREASES IN GENITAL BLOOD  
FLOW IN THE ANAESTHETISED RABBIT MODEL OF SEXUAL AROUSAL

##### 1.0 Aims

To develop and validate an animal model of male sexual arousal.

To identify the mechanism(s) responsible for the regulation of genital blood flow in the anaesthetised rabbit.

To identify potential approaches for enhancement of genital blood flow.

To investigate the mechanism(s) that underlie relaxation of genital smooth muscle and to identify potential approaches for enhancement of relaxation.

##### 2.0 Introduction

The normal sexual arousal response consists of a number of physiological responses that are observed during sexual excitement. These changes result from increases in genital blood flow.

As explained herein, the present invention provides a means for restoring or potentiating the normal sexual arousal response in men suffering from MED, by enhancing genital blood flow.

5 In our studies, we have identified cAMP (cyclic adenosine-3',5'-monophosphate) as a mediator of vasorelaxation using laser Doppler technology to measure small changes in genital blood flow. Using an inhibitor of VIP metabolism (a NEP EC3.4.24.11 inhibitor), we have also demonstrated that the increases in genital blood flow observed during pelvic nerve stimulation (ie sexual arousal) are mediated by VIP.

10 This has involved developing an animal model of sexual arousal and demonstrating that the data reflects the physiological changes observed during male sexual arousal. The model has then been used to identify and validate mechanisms that enhance genital blood flow eg. direct or indirect potentiation of cAMP-mediated vasorelaxation.

### 15 3.0 Methods

#### 3.1 *Anaesthetic Protocol*

Male New Zealand rabbits (~2.5kg) were pre-medicated with a combination of  
20 Medetomidine (Domitor®) 0.5ml/kg *i.m.*, and Ketamine (Vetalar®) 0.25ml/kg *i.m.* whilst maintaining oxygen intake via a face mask. The rabbits were tracheotomised using a Portex™ uncuffed endotracheal tube 3-4mm ID., connected to a ventilator and maintained at a ventilation rate of 30-40 breaths per minute, with an approximate tidal volume of 18-20 ml, and a maximum airway pressure of 10 cm H<sub>2</sub>O.

25 Anaesthesia was then switched to Isoflurane and ventilation continued with O<sub>2</sub> at 0.5L/min. The right marginal ear vein was cannulated using a 23G or 24G catheter, and Lactated Ringer solution perfused at 0.5ml/min. The rabbit was maintained at 3% Isoflurane during invasive surgery, dropping to 2% for maintenance anaesthesia.

#### 30 3.2 *Cannulation of Vessels*

The left jugular vein was exposed, isolated and then cannulated with a PVC catheter (17G) for the infusion of drugs and compounds. The skin around the penis was removed and a Vygon catheter (246.08) was inserted into the left *corpus cavernosum*. This catheter was linked to a Gould system to record intra cavernosal  
35 pressure. Heart rate was monitored via the pulse oxymeter and *Po-ne-mah* data



acquisition software system (Ponemah Physiology Platform, Gould Instrument Systems Inc).

### 3.3 *Stimulation of the Pelvic Nerve*

5

A ventral midline incision was made into the abdominal cavity. The incision was about 5cm in length just above the pubis. The fat and muscle was *bluntly* dissected away to reveal the hypogastric nerve which runs down the body cavity. It was essential to keep close to the side curve of the pubis wall in order to avoid damaging the femoral vein and artery, which lie above the pubis. The sciatic and pelvic nerves lie deeper and were located after further dissection on the dorsal side of the rabbit. Once the sciatic nerve is identified, the pelvic nerve was easily located. The term *pelvic nerve* is loosely applied; anatomy books on the subject fail to identify the nerves in sufficient detail. However, stimulation of the nerve causes an increase in cavernosal blood pressure, and innervation of the pelvic region. The pelvic nerve was freed away from surrounding tissue and a *Harvard* bipolar stimulating electrode was placed around the nerve. The nerve was slightly lifted to give some tension, then the electrode was secured in position. Approximately 1ml of light paraffin oil was placed around the nerve and electrode. This acts as a protective lubricant to the nerve and prevents blood contamination of the electrode. The electrode was connected to a *Grass* S88 Stimulator. The pelvic nerve was stimulated using the following parameters:- 5-20V, pulse width 0.5ms, duration of stimulus 20 seconds and a frequency of 16Hz. Reproducible responses were obtained when the nerve was stimulated every 15-20 minutes.

5

The compound(s) to be tested were infused, via the jugular vein, using a *Harvard* 22 infusion pump allowing a continuous 15-minute stimulation cycle.

### 3.4 *Positioning of the Laser Doppler Probe*

30

A laser Doppler flow probe was positioned on the external corpus cavernosal wall of the exposed penis. The position of this probe was adjusted until a signal was obtained. The probe was then clamped in position.

35

Corpus cavernosal flow was recorded either as numbers directly from the Flowmeter using *Po-ne-mah* data acquisition software (Ponemah Physiology Platform, Gould

Instrument Systems Inc), or indirectly from Gould chart recorder trace. Calibration was set at the beginning of the experiment (0-125ml/min/100g tissue).

### 3.5 *Infusion of Inhibitors*

5

NEP (Neutral Endopeptidase EC3.4.24.11) inhibitors, phosphodiesterase type 5 (PDE5) inhibitors and NPY Y1 antagonists were made up in saline or 5% glucose solution (200µl 50% glucose in 1.8ml water for injection). PDE<sub>CAMP</sub> inhibitors were dissolved in a 40% ethanol solution (200µl 50% glucose in 1.8ml water/ethanol for injection. NEP inhibitors, NPY Y1 receptor antagonists and PDE<sub>CAMP</sub> inhibitors were left for 15 minutes prior to pelvic nerve stimulation.

10

## 4.0 Results and Discussion

15

### 4.1 Animal model of sexual arousal

In our studies, we have developed a robust reproducible model of the physiology of sexual arousal. Using this anaesthetised rabbit model, we are capable of measuring small changes in intracavernosal pressure, and flow using Laser Doppler technology. Stimulation of the pelvic nerve is used to simulate the neuronal effects of sexual arousal.

20

We found that stimulation of the pelvic nerve induces increases in intracavernosal pressure

5

We found that there are no adverse cardiovascular effects associated with pelvic nerve stimulation in the anaesthetised rabbit.

Preliminary results indicate that NPY inhibitors and its receptors, preferably selective NPY1 receptor inhibitors have the effect of potentiating intracavernosal pressure following stimulation of the pelvic nerve.

30

## NEPi – ANIMAL TEST METHODS

35

### Animal models

### Anaesthetised Rabbit Methodology

Male New Zealand rabbits (~2.5kg) were pre-medicated with a combination of Medetomidine (Domitor®) 0.5ml/kg *i.m.*, and Ketamine (Vetalar®) 0.25ml/kg *i.m.* whilst maintaining oxygen intake via a face mask. The rabbits were tracheotomised using a Portex™ uncuffed endotracheal tube 3 ID., connected to ventilator and maintained at a ventilation rate of 30-40 breaths per minute, with an approximate tidal volume of 18-20 ml, and a maximum airway pressure of 10 cm H<sub>2</sub>O. Anaesthesia was then switched to Isoflurane and ventilation continued with O<sub>2</sub> at 2l/min. The right marginal ear vein was cannulated using a 23G or 24G catheter, and Lactated Ringer solution perfused at 0.5ml/min. The rabbit was maintained at 3% Isoflurane during invasive surgery, dropping to 2% for maintenance anaesthesia. The left jugular vein was exposed, isolated and then cannulated with a PVC catheter (17G) for the infusion of drugs and compounds. A ventral midline incision was made into the abdominal cavity. The incision was about 5cm in length just above the pubis. The fat and muscle was *bluntly* dissected away to reveal the hypogastric nerve which runs down the body cavity. It was essential to keep close to the side curve of the pubis wall in order to avoid damaging the femoral vein and artery which lie above the pubis. The sciatic and pelvic nerves lie deeper and were located after further dissection on the dorsal side of the rabbit. Once the sciatic nerve is identified, the pelvic nerve was easily located. The term *pelvic nerve* is loosely applied; anatomy books on the subject fail to identify the nerves in sufficient detail. However, stimulation of the nerve causes an increase in vaginal and clitoral blood flow, and innervation of the pelvic region. The pelvic nerve was freed away from surrounding tissue and a *Harvard* bipolar stimulating electrode was placed around the nerve. The nerve was slightly lifted to give some tension, then the electrode was secured in position. Approximately 1ml of light paraffin oil was placed around the nerve and electrode. This acts as a protective lubricant to the nerve and prevents blood contamination of the electrode. The electrode was connected to a *Grass* S88 Stimulator. The pelvic nerve was stimulated using the following parameters:- 5V, pulse width 0.5ms, duration of stimulus 20 seconds with a frequency of 16Hz. Reproducible responses were obtained when the nerve was stimulated every 15-20 minutes. Several stimulations using the above parameters were performed to establish a mean control response. The compound(s) to be tested were infused, via the jugular vein, using a *Harvard* 22 infusion pump allowing a continuous 15 minute stimulation cycle. The skin and connective tissue around the penis was removed to expose the penis. A catheter set (*Insyte-W*, Becton-Dickinson 20 Gauge 1.1 x

48mm) was inserted through the tunica albica into the left *corpus cavernosal* space and the needle removed, leaving a flexible catheter. This catheter was linked via a pressure transducer (Ohmeda 5299-04) to a Gould system to record intracavernosal pressure. Once an intracavernosal pressure was established, the catheter was sealed in place using *Vetbond* (tissue adhesive, 3M). Heart rate was measured via the pulse oxymeter and *Po-ne-mah* data acquisition software system (Ponemah Physiology Platform, Gould Instrument Systems Inc).

Intracavernosal blood flow was recorded either as numbers directly from the Flowmeter using *Po-ne-mah* data acquisition software (Ponemah Physiology Platform, Gould Instrument Systems Inc), or indirectly from Gould chart recorder trace. Calibration was set at the beginning of the experiment (0-125ml/min/100g tissue). The NEP (Neutral Endopeptidase EC3.4.24.11) inhibitor was made up in saline + 10% 1M NaOH. The inhibitors and vehicle controls were infused at a rate of 0.1ml/second. NEP inhibitors were left for 15 minutes prior to pelvic nerve stimulation.

All data are reported as mean  $\pm$  s.e.m.. Significant changes were identified using Student's t-tests.

#### Anaesthetised Dog Methodology

Male beagles, in the range 12-15 kg body weight, were deprived of food overnight. They were anaesthetised with pentobarbitone (30-45mg/kg *i.v.*), and the anaesthesia maintained by a continual infusion of pentobarbitone (60mg/ml) at a rate of 1-1.4ml/h. The left femoral artery was cannulated for the measurement of blood pressure, lead II E.C.G. was recorded and heart-rate derived. A catheter was introduced into the left femoral vein for the administration of compounds. Both ureters were cannulated *via* a mid-line abdominal incision to prevent urine accumulation in the bladder and the bladder was completely emptied. The left internal pudendal artery was carefully dissected free of surrounding tissues to allow placement of a Transonic flow probe for the measurement of arterial blood flow. The cavernosal branches of both pelvic nerves were dissected free and placed into bipolar stimulating electrodes. The skin around the penis was opened and the corpora cavernosa exposed. A 21g needle, attached by flexible catheter to a pressure transducer, was inserted into the corpus (usually the left) for measurement of both *i.c.* pressure and injection of SNP; the

system was filled with heparinised saline (15 to 20 U/ml). In the dog the corpora are totally separate which enabled either or both sides to be used if necessary.

The dogs were respired with a Ugo Basile 5025 dog ventilator adjusted to maintain blood gasses in the range pO<sub>2</sub> 95-115 mmHg; pCO<sub>2</sub> 25-40 mmHg. Expired air was continually monitored by a Datex Normocap 200 to aid respiratory control. Body temperature was maintained within the range 36-38°C using an electric blanket. Parameters were recorded on a Gould TA4000 polygraph and all data acquisition and calculation of derived parameters was carried out on-line using a Po-Ne-Mah system. The cavernosal branches of the pelvic nerves were stimulated with a Grass S88 stimulator at 10 volts, 2 ms duration for <1 min. At the end of the experiment dogs were killed by an *i.v.* injection of 20ml saturated potassium chloride, whilst still under pentobarbitone anaesthesia. Following a period of equilibration, the pelvic nerves were stimulated at 16Hz in order to assess whether the rise in *i.c.* pressure was rapidly and fully registered by the transducer and changes in blood flow were detected. Control responses were obtained to nerve stimulation at either 1 or 2Hz, On recovery a second stimulation was performed, at double the first frequency. In some dogs a third frequency was used. This stimulation cycle was repeated after 30 min. NEP inhibitors were dissolved in alkaline saline and given as a series of two-tiered infusions starting with a loading infusion and a maintenance infusion for 30 minutes, when the second set of infusions was started. Subsequent infusions were started either at 30 min intervals or when *i.c.* pressure had returned to baseline. All Infusions were given at a rate of 1ml/min. Stimulation cycles were started fifteen minutes into each infusion.

In addition, arterial blood samples were taken from the abdominal aorta, *via* the blood pressure cannula, pre-dose and at 15 and 30minutes into each infusion, for subsequent analysis of unbound compound concentration by Drug Metabolism.

#### NEP ENZYME ASSAY

THE PREPARATION AND ASSAY OF SOLUBLE (NEP) NEUTRAL ENDOPEPTIDASE FROM CANINE, RAT, RABBIT AND HUMAN KIDNEY CORTEX.

Soluble NEP is obtained from the kidney cortex and activity is assayed by measuring the rate of cleavage of the NEP substrate Abz-D-Arg-Arg-Leu-EDDnp to generate its fluorescent product, Abz-D-Arg-Arg.

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## EXPERIMENTAL PROCEDURE:-

### **1. MATERIALS**

All water is double de ionised.

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#### **1.1 Tissues**

Human Kidney IIAM (Pennsylvania. U.S.A.)

Rat Kidney

Rabbit Kidney

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Canine Kidney

#### **1.2 Homogenisation medium**

100mM Mannitol and 20mM Tris @ pH 7.1

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2.42g Tris (Fisher T/P630/60) is diluted in 1 litre of water and the pH adjusted to 7.1 using 6M HCl at room temperature. To this 18.22g Mannitol (Sigma M-9546) is added.

#### **1.3 Tris buffer (NEP buffer).**

50ml of 50mM Tris pH 7.4 (Sigma T2663) is diluted in 950ml of water.

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#### **1.4 Substrate (Abz-D-Arg-Arg-Leu-EDDnp)**

Made to order from SNPE, and is stored as a powder at  $-20^{\circ}\text{C}$ . A 2mM stock is made by gently re-suspending the substrate in Tris buffer, this should not be vortexed or sonicated. 600 $\mu\text{l}$  aliquots of the 2mM stock are stored at  $-20$  for up to one month. (Medeiros, M.A.S., Franca, M.S.F. et al., (1997), Brazilian Journal of Medical and Biological Research, 30, 1157-1162).

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#### **1.5 Total product**

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Samples corresponding to 100% substrate to product conversion are included on the plate to enable the % substrate turnover to be determined. The total product is generated by incubating 1ml of 2mM substrate with 20 $\mu\text{l}$  of enzyme stock for 24 hours at  $37^{\circ}\text{C}$ .

### 1.6 Stop solution.

A 300µM stock of Phosphoramidon (Sigma R7385) is made up in NEP buffer and stored in 50µl aliquots at -20.

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### 1.7 Dimethyl sulphoxide (DMSO).

### 1.8 Magnesium Chloride -MgCl<sub>2</sub>.6H<sub>2</sub>O (Fisher M0600/53).

### 1.9 Black 96 well flat bottom assay plates (Costar 3915).

### 1.10 Topseal A (Packard 6005185).

### 10 1.11 Centrifuge tubes

## 2. SPECIFIC EQUIPMENT

### 2.1 Sorvall RC-5B centrifuge (SS34 GSA rotor, pre-cooled to 4°C).

### 2.2 Braun miniprimer mixer.

### 15 2.3 Beckman CS-6R centrifuge.

### 2.4 Fluostar galaxy.

### 2.5 Wesbart 1589 shaking incubator.

## 3. METHODS

### 20 3.1 TISSUE PREPARATION

3.2 Dog, rat, rabbit, and human NEP is obtained from the kidney cortex using a method adapted from Booth, A.G. & Kenny, A.J. (1974) *Biochem. J.* 142, 575-581.

3.3 Frozen kidneys are allowed to thaw at room temperature and the cortex is dissected away from the medulla.

5 3.4 The cortex is finely chopped and homogenised in approximately 10 volumes of homogenisation buffer (1.2) using a Braun miniprimer (2.2).

3.5 Magnesium chloride (1.8) (20.3mg/gm tissue) is added to the homogenate and stirred in an ice-water bath for 15 minutes.

3.6 The homogenate is centrifuged at 1,500g (3,820rpm) for 12 minutes in a Beckman centrifuge (2.3) before removing the supernatant to a fresh centrifuge tube and discarding the pellet.

3.7 The supernatant is centrifuged at 15,000g (12,100rpm) for 12 minutes in a Sovall centrifuge (2.1) and the supernatant is discarded.

3.8 The pale pink layer on the top of the remaining pellet is removed and re-suspended in homogenisation buffer containing magnesium chloride (9mg MgCl in 5ml buffer per 1g tissue).

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3.9 The suspension is centrifuged at 2,200g (4,630rpm) for 12 minutes in a Beckman centrifuge (2.3) before discarding the pellet.

3.10 The supernatant is centrifuged at 15,000g (12,100rpm) for 12 minutes using the Sorvall centrifuge (2.1) and the supernatant is discarded.

5 3.11 The final pellet is resuspended in homogenisation buffer containing magnesium chloride (0.9mg MgCl in 0.5ml buffer per 1g tissue). A homogenous suspension is obtained using a Braun miniprimer (2.2). This is then frozen down in 100µl aliquots to be assayed for NEP activity.

#### 10 4.0 DETERMINATION OF NEP ACTIVITY

The activity of the previously aliquoted NEP is measured by its ability to cleave the NEP specific peptide substrate.

15 4.1 A 4% DMSO/NEP buffer solution is made (4mls DMSO in 96mls NEP buffer).

4.2 Substrate, total product, enzyme, and Phosphoramidon stocks are left on ice to thaw.

4.3 50µl of 4% DMSO/NEP buffer solution is added to each well.

20 4.4 The 2mM substrate stock is diluted 1:40 to make a 50µM solution. 100µl of 50µM substrate is added to each well (final concentration 25µM).

4.5 50µl of a range of enzyme dilutions is added to initiate the reaction (usually 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200 are used). 50µl of NEP buffer is added to blank wells.

25 4.6 The 2mM total product is diluted 1:80 to make a 25µM solution. 200µl of 25µM product is added to the first four wells of a new plate.

4.7 Plates are incubated at 37°C in a shaking incubator for 60 minutes.

4.8 The 300µM Phosphoramidon stock is diluted 1:100 to 300nM. The reaction is stopped by the addition of 100µl 300nM Phosphoramidon and incubated at 37°C in a shaking incubator for 20 minutes before being read on the Fluostar (ex320/em420).

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#### 5. NEP INHIBITION ASSAYS

35 5.1 Substrate, total product, enzyme and Phosphoramidon stocks are left on ice to thaw.



5.2 Compound stocks are made up in 100% DMSO and diluted 1:25 in NEP buffer to give a 4% DMSO solution. All further dilutions are carried out in a 4% DMSO solution (4mls DMSO in 96mls NEP buffer).

5.3 50µl of compound in duplicate is added to the 96 well plate and 50µl of 4% DMSO/NEP buffer is added to control and blank wells.

5.4 The 2mM substrate stock is diluted 1:40 in NEP buffer to make a 50µM solution (275µl 2mM substrate to 10.73ml buffer is enough for 1 plate).

5.5 The enzyme stock diluted in NEP buffer (determined from activity checks).

5.6 The 2mM total product stock is diluted 1:80 in NEP buffer to make a 25µM solution. 200µl is added to the first four wells of a separate plate.

5.7 The 300µM Phosphoramidon stock is diluted 1:1000 to make a 300nM stock (11µl Phosphoramidon to 10.99ml NEP buffer).

5.8 To each well in the 96 well plate the following is added:

Table Reagents to be added to 96 well plate.

	Compound/ DMSO	Tris Buffer	Substrate	NEP enzyme	Total product
Samples	2µl compound	50µl	100µl	50µl	None
Controls	2µl DMSO	50µl	100µl	50µl	None
Blanks	2µl DMSO	100µl	100µl	None	None
Totals	2µl DMSO	None	None	None	200µl

5.9 The reaction is initiated by the addition of the NEP enzyme before incubating at 37°C for 1 hour in a shaking incubator.

5.10 The reaction is stopped with 100µl 300nM Phosphoramidon and incubated at 37°C for 20 minutes in a shaking incubator before being read on the Fluostar (ex320/em420).

## 6. CALCULATIONS

The activity of the NEP enzyme is determined in the presence and absence of compound and expressed as a percentage.

% Control activity (turnover of enzyme):

$$\frac{\text{Mean FU of controls} - \text{Mean FU of blanks}}{\text{Mean FU of totals} - \text{Mean FU of blanks}} \times 100$$

5 % Activity with inhibitor:

$$\frac{\text{Mean FU of compound} - \text{Mean FU of blanks}}{\text{Mean FU of totals} - \text{Mean FU of blanks}} \times 100$$

10 Activity expressed as % of control:

$$\frac{\text{% Activity with inhibitor}}{\text{% Control activity}} \times 100$$

15 A sigmoidal dose-response curve is fitted to the % activities (% of control) vs compound concentration and IC50 values calculated using LabStats fit-curve in Excel.

### ACE ASSAY

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THE PREPARATION AND ASSAY OF SOLUBLE ANGIOTENSIN CONVERTING ENZYME (ACE), FROM PORCINE AND HUMAN KIDNEY CORTEX.

Soluble ACE activity is obtained from the kidney cortex and assayed by measuring the rate of cleavage of the ACE substrate Abz-Gly-p-nitro-Phe-Pro-OH to generate its fluorescent product, Abz-Gly.

#### 1. MATERIALS

30 All water is double de ionised.

1.1 Human Kidney IIAM (Pennsylvania. U.S.A.) or UK Human Tissue Bank (UK HTB)

1.2 Porcine kidney ACE Sigma (A2580)

35 1.3 Homogenisation buffer-1

100mM Mannitol and 20mM Tris @ pH 7.1

2.42g Tris (Fisher T/P630/60) is diluted in 1 litre of water and the pH adjusted to 7.1 using 6M HCl at room temperature. To this 18.22g Mannitol (Sigma M-9546) is added.

#### 1.4 Homogenisation buffer-2

100mM Mannitol, 20mM Tris @ pH7.1 and 10mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (Fisher M0600/53)

To 500ml of the homogenisation buffer 1 (1.4) 1.017g of  $\text{MgCl}_2$  is added.

#### 1.5 Tris buffer (ACE buffer).

5 50mM Tris and 300mM NaCl @ pH 7.4

50ml of 50mM Tris pH 7.4 (Sigma T2663) and 17.52g NaCl (Fisher S/3160/60) are made up to 1000ml in water.

#### 1.6 Substrate (Abz-D-Gly-p-nitro-Phe-Pro-OH) (Bachem M-1100)

ACE substrate is stored as a powder at  $-20^\circ\text{C}$ . A 2mM stock is made by gently re-

10 suspending the substrate in ACE buffer, this must not be vortexed or sonicated.

400 $\mu\text{l}$  aliquots of the 2mM stock are stored at  $-20^\circ\text{C}$  for up to one month.

#### 1.7 Total product

Samples corresponding to 100% substrate to product conversion are included on the plate to enable the % substrate turnover to be determined (see calculations). The

15 total product is generated by incubating 1ml of 2mM substrate with 20 $\mu\text{l}$  of enzyme stock for 24 hours at  $37^\circ\text{C}$ .

#### 1.8 Stop solution.

0.5M EDTA (Promega CAS[6081/92/6]) is diluted 1:250 in ACE buffer to make a 2mM solution.

20 1.9 Dimethyl sulphoxide (DMSO).

1.10 Magnesium Chloride  $-\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (Fisher M0600/53).

1.11 Black 96 well flat bottom assay plates (Costar 3915 or Packard).

1.12 Topseal A (Packard 6005185).

1.13 Centrifuge tubes

## 2. SPECIFIC EQUIPMENT

2.1 Sorvall RC-5B centrifuge (SS34 GSA rotor, pre-cooled to  $4^\circ\text{C}$ ).

2.2 Braun miniprimer mixer.

30 2.3 Beckman CS-6R centrifuge.

2.4 BMG Fluostar Galaxy.

2.5 Wesbart 1589 shaking incubator.

## 3. METHODS

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### 3.1 TISSUE PREPARATION

3.3 Human ACE is obtained from the kidney cortex using a method adapted from Booth, A.G. & Kenny, A.J. (1974) *Biochem. J.* 142, 575-581.

3.3 Frozen kidneys are allowed to thaw at room temperature and the cortex is dissected away from the medulla.

5 3.4 The cortex is finely chopped and homogenised in approximately 10 volumes of homogenisation buffer-1 (1.4) using a Braun miniprimer (2.2).

3.5 Magnesium chloride (1.11) (20.3mg/gm tissue) is added to the homogenate and stirred in an ice-water bath for 15 minutes.

10 3.6 The homogenate is centrifuged at 1,500g (3,820rpm) for 12 minutes in a Beckman centrifuge (2.3) before removing the supernatant to a fresh centrifuge tube and discarding the pellet.

3.7 The supernatant is centrifuged at 15,000g (12,100rpm) for 12 minutes in a Sovall centrifuge (2.1) and the supernatant is discarded.

15 3.8 The pale pink layer on the top of the remaining pellet is removed and re-suspended in homogenisation buffer-2 (1.5) (5ml buffer per 1g tissue).

3.9 The suspension is centrifuged at 2,200g (4,630rpm) for 12 minutes in a Beckman centrifuge before discarding the pellet.

3.10 The supernatant is centrifuged at 15,000g (12,100rpm) for 12 minutes using the Sorvall centrifuge and the supernatant is discarded.

20 3.11 The final pellet is resuspended in homogenisation buffer-2 (0.5ml buffer per 1g tissue). A homogenous suspension is obtained using a Braun miniprimer. This is then frozen down in 100µl aliquots to be assayed for NEP activity.

#### 4.0 DETERMINATION OF ACE ACTIVITY

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The activity of the previously aliquoted ACE is measured by its ability to cleave the ACE specific peptide substrate.

Porcine ACE (1.2) is defrosted and resuspended in ACE buffer (1.6) at 0.004U/µl, this is frozen down in 50µl aliquots.

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4.1 A 4% DMSO/ACE buffer solution is made (4mls DMSO in 96mls ACE buffer).

4.2 Substrate (1.7), total product (1.8) and enzyme (1.1, 1.2, 1.3), are left on ice to thaw.

4.3 50µl of 4% DMSO/ACE buffer solution is added to each well.

35 4.4 The 2mM substrate stock is diluted 1:100 to make a 20µM solution. 100µl of 20µM substrate is added to each well (final concentration in the assay 10µM).

4.5 50µl of a range of enzyme dilutions is added to initiate the reaction (usually 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200 are used). 50µl of ACE buffer is added to blank wells.

4.6 The 2mM total product is diluted 1:200 to make 10µM solution. 200µl 10µM product is added to the first four wells of a new plate.

4.7 Plates are incubated at 37°C in a shaking incubator for 60 minutes.

4.8 The enzyme reaction is stopped by the addition of 100µl 2mM EDTA in ACE buffer and incubated at 37°C in a shaking incubator for 20 minutes before being read on the BMG Fluostar Galaxy (ex320/em420).

## 5. ACE INHIBITION ASSAYS

5.1 Substrate, total product, and enzyme stocks are left on ice to thaw.

5.2 Compound stocks are made up in 100% DMSO and diluted 1:25 in ACE buffer to give a 4% DMSO solution. All further dilutions are carried out in a 4% DMSO/ACE buffer solution (4mls DMSO in 96mls ACE buffer).

5.3 50µl of compound, in duplicate, is added to the 96 well plate and 50µl of 4% DMSO/ACE buffer is added to control and blank wells.

5.4 Steps 5.2 and 5.3 can be carried out either by hand or using the Packard multiprobe robots

5.5 The 2mM substrate stock is diluted 1:100 in ACE buffer to make a 20µM solution (10µM final concentration in the assay) (110µl of 2mM substrate added to 10.89ml buffer is enough for 1 plate).

5.6 The enzyme stock is diluted in ACE buffer, as determined from activity checks (4.0).

5.7 The 2mM total product stock is diluted 1:200 in ACE buffer to make a 10µM solution. 200µl is added to the first four wells of a separate plate.

5.8 The 0.5mM EDTA stock is diluted 1:250 to make a 2mM stock (44µl EDTA to 10.96ml ACE buffer).

5.9 To each well of the 96 well plate the following reagents are added:

Table 1: Reagents added to 96 well plate.

	Compound/ DMSO	Tris Buffer	Substrate	ACE enzyme	Total product
Samples	2µl compound	50µl	100µl	50µl	None

Controls	2µl DMSO	50µl	100µl	50µl	None
Blanks	2µl DMSO	100µl	100µl	None	None
Totals	2µl DMSO	None	None	None	200µl

5.10 50µl of the highest concentration of each compound used in the assay is added in duplicate to the same 96 well plate as the totals (5.7). 150µl of ACE buffer is added to determine any compound fluorescence.

5.11 The reaction is initiated by the addition of the ACE enzyme before incubating at 37°C for 1 hour in a shaking incubator.

5.12 The reaction is stopped by the addition of 100µl 2mM EDTA and incubated at 37°C for 20 minutes in a shaking incubator, before being read on the BMG Fluostar Galaxy (ex320/em420).

## 6. CALCULATIONS

The activity of the ACE enzyme is determined in the presence and absence of compound and expressed as a percentage.

FU = Fluorescence units

(i) % Control activity (turnover of enzyme):

$$\frac{\text{Mean FU of controls} - \text{Mean FU of blanks}}{\text{Mean FU of totals} - \text{Mean FU of blanks}} \times 100$$

(ii) % Activity with inhibitor:

$$\frac{\text{Mean FU of compound} - \text{Mean FU of blanks}}{\text{Mean FU of totals} - \text{Mean FU of blanks}} \times 100$$

(iii) Activity expressed as % of control:

$$\frac{\text{\% Activity with inhibitor}}{\text{\% Control activity}} \times 100$$

$$\text{OR } \frac{\text{Mean FU of compound} - \text{Mean FU of blanks}}{\text{Mean FU of controls} - \text{Mean FU of blanks}} \times 100$$

(iv) % Inhibition = 100 - % control

(v) For fluorescent compounds the mean FU of blanks containing compound (5.10) is deducted from the mean FU of compound values used to calculate the % Activity.

A sigmoidal dose-response curve is fitted to the % activities (% of control) vs compound concentration and IC<sub>50</sub> values calculated using LabStats fit-curve in Excel.

#### PDE5 inhibitor – TEST METHODS

##### Phosphodiesterase (PDE) inhibitory activity

Preferred in combination with the NPY inhibitors of the present invention are cGMP PDE5 inhibitors. In vitro PDE inhibitory activities against cyclic guanosine 3',5'-monophosphate (cGMP) and cyclic adenosine 3',5'-monophosphate (cAMP) phosphodiesterases were determined by measurement of their IC<sub>50</sub> values (the concentration of compound required for 50% inhibition of enzyme activity).

The required PDE enzymes were isolated from a variety of sources, including human corpus cavernosum, human and rabbit platelets, human cardiac ventricle, human skeletal muscle and human and canine retina, essentially by the method of W.J. Thompson and M.M. Appleman (Biochem., 1971, 10, 311). In particular, the cGMP-specific PDE (PDE5) and the cGMP-inhibited cAMP PDE (PDE3) were obtained from human corpus cavernosum or human platelets; the cGMP-stimulated PDE (PDE2) was obtained from human corpus cavernosum and human platelets; the calcium/calmodulin (Ca/CAM)-dependent PDE (PDE1) from human cardiac ventricle; the cAMP-specific PDE (PDE4) from human skeletal muscle and human recombinant, expressed in SF9 cells; and the photoreceptor PDE (PDE6) from human or canine retina. Phosphodiesterases 7-11 were generated from full length human recombinant clones transfected into SF9 cells.

Assays can be performed either using a modification of the "batch" method of W.J. Thompson et al. (Biochem., 1979, 18, 5228) or using a scintillation proximity

assay for the direct detection of AMP/GMP using a modification of the protocol described by Amersham plc under product code TRKQ7090/7100. In summary, the effect of PDE inhibitors was investigated by assaying a fixed amount of enzyme in the presence of varying inhibitor concentrations and low substrate, (cGMP or cAMP in a 3:1 ratio unlabelled to [<sup>3</sup>H]-labelled at a conc ~1/3  $K_m$ ) such that  $IC_{50} \cong K_i$ . The final assay volume was made up to 100µl with assay buffer [20 mM Tris-HCl pH 7.4, 5 mM MgCl<sub>2</sub>, 1 mg/ml bovine serum albumin]. Reactions were initiated with enzyme, incubated for 30-60 min at 30°C to give <30% substrate turnover and terminated with 50 µl yttrium silicate SPA beads (containing 3 mM of the respective unlabelled cyclic nucleotide for PDEs 9 and 11). Plates were re-sealed and shaken for 20 min, after which the beads were allowed to settle for 30 min in the dark and then counted on a TopCount plate reader (Packard, Meriden, CT) Radioactivity units were converted to % activity of an uninhibited control (100%), plotted against inhibitor concentration and inhibitor  $IC_{50}$  values obtained using the 'Fit Curve' Microsoft Excel extension (or in-house equivalent). Results from these tests show that the compounds of the present invention are inhibitors of cGMP-specific PDE5.

Preferred have  $IC_{50}$  values of less than about 10nM for the PDE5 enzyme. A further preferred group of compounds have  $IC_{50}$  values of less than about 5nM for the PDE5 enzyme.

Highly preferred herein are compounds which have an  $IC_{50}$  value of less than about 10M, more preferably less than about 5nM for the PDE5 enzyme in combination with greater than 2-fold, preferably greater than 3-fold selectivity for the PDE5 enzyme versus the PDE6 enzyme.

#### Functional activity

This can be assessed in vitro by determining the capacity of a compound of the invention to enhance sodium nitroprusside or electrical field stimulation-induced relaxation of pre-contracted rabbit corpus cavernosum tissue strips, using methods based on that described by S.A. Ballard et al. (Brit. J. Pharmacol., 1996, 118 (suppl.), abstract 153P) or S.A. Ballard et al. (J. Urology, 1998, 159, 2164-2171).

#### In vivo activity

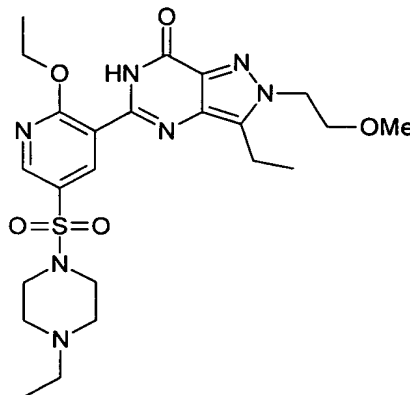


Compounds can be screened in anaesthetised dogs to determine their capacity, after i.v. administration, to enhance the pressure rises in the corpora cavernosa of the penis induced by intracavernosal injection of sodium nitroprusside, using a method based on that described by Trigo-Rocha *et al.* (Neurourol. and Urodyn., 1994, 13, 71).

## CHEMICAL COMPOUND EXAMPLES and Preparations

### PDE5i Example 1

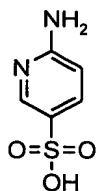
2-(Methoxyethyl)-5-[2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one



A mixture of the product from stage j) below (0.75mmol), potassium bis(trimethylsilyl)amide (298mg, 1.50mmol) and ethyl acetate (73 microlitres, 0.75mmol) in ethanol (10ml) was heated at 120°C in a sealed vessel for 12 hours. The cooled mixture was partitioned between ethyl acetate and aqueous sodium bicarbonate solution, and the layers separated. The organic phase was dried (MgSO<sub>4</sub>), and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane:methanol (98:2) as eluant to afford the title compound, 164mg; Found : C, 53.18; H, 6.48; N, 18.14; C<sub>23</sub>H<sub>33</sub>N<sub>7</sub>O<sub>5</sub>S; 0.20C<sub>2</sub>H<sub>5</sub>CO<sub>2</sub>CH<sub>3</sub> requires C, 53.21; H, 6.49; N, 18.25%;  $\delta$  (CDCl<sub>3</sub>) : 1.04 (3H, t), 1.40 (3H, t), 1.58 (3H, t), 2.41 (2H, q), 2.57 (4H, m), 3.08 (2H, q), 3.14 (4H, m), 3.30 (3H, s), 3.92 (2H, t), 4.46 (2H, t), 4.75 (2H, q), 8.62 (1H, d), 9.04 (1H, d), 10.61 (1H, s); LRMS : m/z 520 (M+1)<sup>+</sup>; mp 161-162°C.

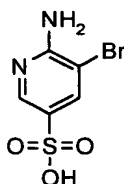
### Preparation of Starting Materials for PDE5i Example 1

a) Pyridine-2-amino-5-sulphonic acid



2-Aminopyridine (80g, 0.85mol) was added portionwise over 30 minutes to oleum (320g) and the resulting solution heated at 140°C for 4 hours. On cooling, the reaction was poured onto ice (200g) and the mixture stirred in an ice/salt bath for a further 2 hours. The resulting suspension was filtered, the solid washed with ice water (200ml) and cold IMS (200ml) and dried under suction to afford the title compound as a solid, 111.3g; LRMS : m/z 175 (M+1)<sup>+</sup>.

b) Pyridine-2-amino-3-bromo-5-sulphonic acid

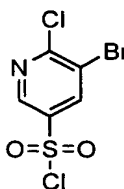


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Bromine (99g, 0.62mol) was added dropwise over an hour, to a hot solution of the product from stage a) (108g, 0.62mol) in water (600ml) so as to maintain a steady reflux. Once the addition was complete the reaction was cooled and the resulting mixture filtered. The solid was washed with water and dried under suction to afford the title compound, 53.4g;  $\delta$  (DMSO-d<sub>6</sub>, 300MHz): 8.08 (1H, s), 8.14 (1H, s); LRMS : m/z 253 (M)<sup>+</sup>.

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c) Pyridine-3-bromo-2-chloro-5-sulphonyl chloride



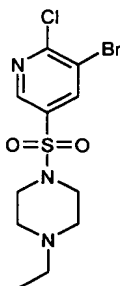
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A solution of sodium nitrite (7.6g, 110.0mmol) in water (30ml) was added dropwise to an ice-cooled solution of the product from stage b) (25.3g, 100.0mmol) in aqueous hydrochloric acid (115ml, 20%), so as to maintain the temperature below 6°C. The reaction was stirred for 30 minutes at 0°C and for a further hour at room temperature. The reaction mixture was evaporated under reduced pressure and the residue dried under vacuum at 70°C for 72 hours. A mixture of this solid, phosphorus pentachloride (30.0g, 144mmol) and phosphorus oxychloride (1ml, 10.8mmol) was heated at 125°C

25

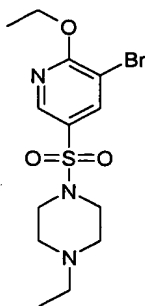
for 3 hours, and then cooled. The reaction mixture was poured onto ice (100g) and the resulting solid filtered, and washed with water. The product was dissolved in dichloromethane, dried ( $\text{MgSO}_4$ ), and evaporated under reduced pressure to afford the title compound as a yellow solid, 26.58g;  $\delta$  ( $\text{CDCl}_3$ , 300MHz) : 8.46 (1H, s), 8.92 (1H, s).

d) 3-Bromo-2-chloro-5-(4-ethylpiperazin-1-ylsulphonyl)pyridine



A solution of 1-ethylpiperazine (11.3ml, 89.0mmol) and triethylamine (12.5ml, 89.0mmol) in dichloromethane (150ml) was added dropwise to an ice-cooled solution of the product from stage c) (23.0g, 79.0mmol) in dichloromethane (150ml) and the reaction stirred at 0°C for an hour. The reaction mixture was concentrated under reduced pressure and the residual brown oil was purified by column chromatography on silica gel, using an elution gradient of dichloromethane:methanol (99:1 to 97:3) to afford the title compound as an orange solid, 14.5g;  $\delta$  ( $\text{CDCl}_3$ , 300MHz) : 1.05 (3H, t), 2.42 (2H, q), 2.55 (4H, m), 3.12 (4H, m), 8.24 (1H, s), 8.67 (1H, s).

e) 3-Bromo-2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridine

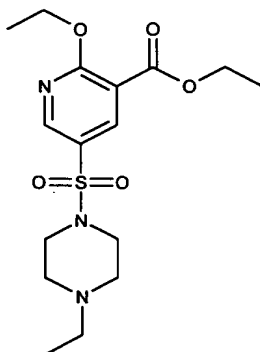


A mixture of the product from stage d) (6.60g, 17.9mmol) and sodium ethoxide (6.09g, 89.55mmol) in ethanol (100ml) was heated under reflux for 18 hours, then cooled. The reaction mixture was concentrated under reduced pressure, the residue partitioned between water (100ml) and ethyl acetate (100ml), and the layers separated. The aqueous phase was extracted with ethyl acetate (2x100ml), the combined organic solutions dried ( $\text{MgSO}_4$ ) and evaporated under reduced pressure

to afford the title compound as a brown solid, 6.41g; Found : C, 41.27; H, 5.33; N, 11.11.  $C_{13}H_{20}BrN_3O_3S$  requires C, 41.35; H, 5.28; N, 10.99%;  $\delta$  ( $CDCl_3$ , 300MHz) : 1.06 (3H, t), 1.48 (3H, t), 2.42 (2H, q), 2.56 (4H, m), 3.09 (4H, m), 4.54 (2H, q), 8.10 (1H, s), 8.46 (1H, s); LRMS : m/z 378, 380 ( $M+1$ )<sup>+</sup>.

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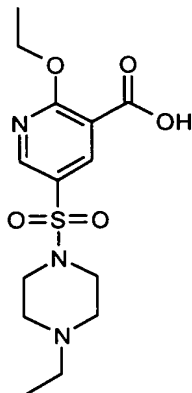
- f) Pyridine 2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)-3-carboxylic acid ethyl ester



A mixture of the product from stage e) (6.40g, 16.92mmol), triethylamine (12ml, 86.1mmol), and palladium (0) tris(triphenylphosphine) in ethanol (60ml) was heated at 100°C and 200 psi, under a carbon monoxide atmosphere, for 18 hours, then cooled. The reaction mixture was evaporated under reduced pressure and the residue purified by column chromatography on silica gel, using an elution gradient of dichloromethane:methanol (100:0 to 97:3) to afford the title compound as an orange oil, 6.2g;  $\delta$  ( $CDCl_3$ , 300MHz) : 1.02 (3H, t), 1.39 (3H, t), 1.45 (3H, t), 2.40 (2H, q), 2.54 (4H, m), 3.08 (4H, m), 4.38 (2H, q), 4.55 (2H, q), 8.37 (1H, s), 8.62 (1H, s); LRMS : m/z 372 ( $M+1$ )<sup>+</sup>.

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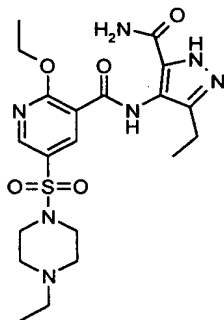
- g) Pyridine 2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)-3-carboxylic acid



20

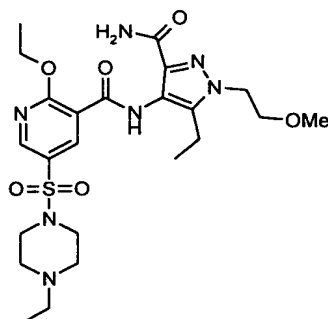
A mixture of the product from stage f) (4.96g, 13.35mmol) and aqueous sodium hydroxide solution (25ml, 2N, 50.0mmol) in ethanol (25ml) was stirred at room temperature for 2 hours. The reaction mixture was concentrated under reduced pressure to half it's volume, washed with ether and acidified to pH 5 using 4N hydrochloric acid. The aqueous solution was extracted with dichloromethane (3x30ml), the combined organic extracts dried (MgSO<sub>4</sub>) and evaporated under reduced pressure to afford the title compound as a tan coloured solid, 4.02g;  $\delta$  (DMSO-d<sub>6</sub>, 300MHz) : 1.18 (3H, t), 1.37 (3H, t), 3.08 (2H, q), 3.17-3.35 (8H, m), 4.52 (2H, q), 8.30 (1H, s), 8.70 (1H, s).

h) 4-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-ylcarboxamido]-1H-3-ethylpyrazole-5-carboxamide



A solution of 4-amino-3-ethyl-1H-pyrazole-5-carboxamide (WO 9849166, preparation 8) (9.2g, 59.8mmol) in N,N-dimethylformamide (60ml) was added to a solution of the product from stage g) (21.7g, 62.9mmol), 1-hydroxybenzotriazole hydrate (10.1g, 66.0mmol) and triethylamine (13.15ml, 94.3mmol) in dichloromethane (240ml). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (13.26g, 69.2mmol) was added and the reaction stirred at room temperature for 6 hours. The dichloromethane was removed under reduced pressure, the remaining solution poured into ethyl acetate (400ml), and this mixture washed with aqueous sodium bicarbonate solution (400ml). The resulting crystalline precipitate was filtered, washed with ethyl acetate and dried under vacuum, to afford the title compound, as a white powder, 22g;  $\delta$  (CDCl<sub>3</sub>+1 drop DMSO-d<sub>6</sub>) 0.96 (3H, t), 1.18 (3H, t), 1.50 (3H, t), 2.25-2.56 (6H, m), 2.84 (2H, q), 3.00 (4H, m), 4.70 (2H, q), 5.60 (1H, br s), 6.78 (1H, br s), 8.56 (1H, d), 8.76 (1H, d), 10.59 (1H, s), 12.10-12.30 (1H, s); LRMS: m/z 480 (M+1)<sup>+</sup>.

i) 2-Methoxyethyl-4-[2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-ylcarboxamido]-3-ethylpyrazole-5-carboxamide



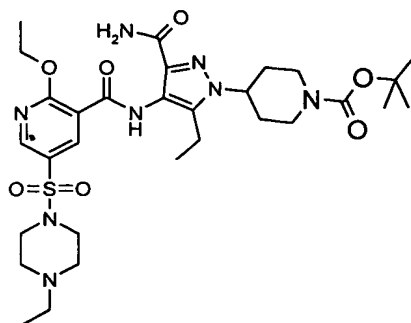
2-ethoxy-5-(4-ethyl-1-piperazinylsulfonyl)nicotinic acid (2.31 kg, 6.73 Mol) was suspended in ethyl acetate (16.2 L) and 1,1-carbonyldimidazole (1.09 kg, 6.73 Mol) was added at room temperature. The reaction mixture was heated at 45°C for 40 minutes and then the reaction was stirred for a further 40 minutes at reflux. After cooling to ambient temperature 4-amino-5-ethyl-1-(2-methoxyethyl)-1H-pyrazole-3-carboxamide (1.5 kg, 7.06 Mol) was added to the cooled mixture, and the reaction stirred for a further 15 hours under reflux. The mixture was cooled filtered and the filter cake was washed with 90% water / 10% ethyl acetate, (2 mL /g) to afford *N*-[3-carbamoyl-5-ethyl-1-(2-methoxyethyl)-1H-pyrazol-4-yl]-2-ethoxy-5-(4-ethyl-1-piperazinyl sulfonyl) nicotinamide as an off white crystalline solid, 3.16 kg, 88%. m.p. = 156°C. Found: C, 51.33; H, 6.56; N, 18.36. C<sub>23</sub>H<sub>35</sub>N<sub>7</sub>O<sub>6</sub>S requires C, 51.40; H, 6.53; N, 18.25%.

$\delta$ (CDCl<sub>3</sub>): 1.04 (3H, t), 1.22 (3H, t), 1.60 (3H, t), 2.44 (2H, q), 2.54 (4H, m), 2.96 (2H, q), 3.12 (4H, m), 3.36 (3H, s), 3.81 (2H, t), 4.27 (2H, t), 4.80(2H, q), 5.35(1H, s), 6.68 (1H, s), 8.66 (1H, d), 8.86 (1H, d), 10.51 (1H, s).

LRMS: m/z = 539 (M+1)<sup>+</sup>

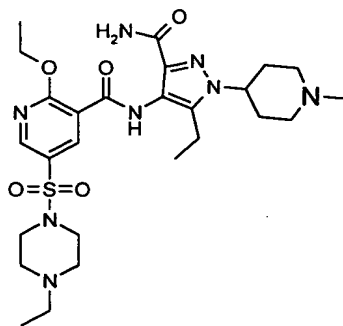
#### Preparation of Starting Materials for PDE5i Example 2

a) 2-(1-*tert*-Butoxycarbonylpiperidin-4-yl)-4-[2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-ylcarboxamido]-3-ethylpyrazole-5-carboxamide



Sodium hydride (64mg, 60% dispersion in mineral oil, 1.6mmol) was added to a solution of the product from Example 1, stage h) (1.46mmol) in tetrahydrofuran (10ml), and the solution stirred for 10 minutes. *tert*-Butyl 4-[(methanesulfonyl)oxy]-1-piperidinecarboxylate (WO 9319059) (1.60mmol) was added and the reaction stirred at 60°C for 3 days. The cooled mixture was partitioned between ethyl acetate and aqueous sodium bicarbonate solution, and the phases separated. The aqueous layer was extracted with ethyl acetate, the combined organic solutions dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using dichloromethane:methanol (98:2) as eluant to afford the title compound as a white foam, 310 mg;  $\delta$  (CDCl<sub>3</sub>) 1.02 (3H, t), 1.23 (3H, t), 1.49 (9H, s), 1.57 (3H, m), 1.93 (2H, m), 2.16 (2H, m), 2.40 (2H, q), 2.54 (4H, m), 2.82-2.97 (4H, m), 3.10 (4H, m), 4.30 (3H, m), 4.79 (2H, q), 5.23 (1H, s), 6.65 (1H, s), 8.63 (1H, d), 8.82 (1H, d), 10.57 (1H, s).

b) 4-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-ylcarboxamido]-3-ethyl-2-(1-methylpiperidin-4-yl)pyrazole-5-carboxamide

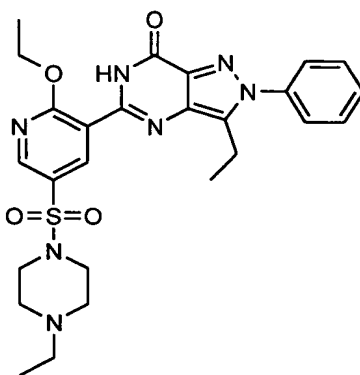


Trifluoroacetic acid (1.5ml) was added to a solution of the product from stage a) above (320mg, 0.48mmol) in dichloromethane (2ml) and the solution stirred at room temperature for 2 ½ hours. The reaction mixture was evaporated under reduced pressure and the residue triturated well with ether and dried under vacuum, to provide a white solid. Formaldehyde (217 microlitres, 37% aqueous, 2.90mmol) was added to a solution of the intermediate amine in dichloromethane (8ml), and the

solution stirred vigorously for 30 minutes. Acetic acid (88 microlitres, 1.69mmol) was added, the solution stirred for a further 30 minutes, then sodium triacetoxyborohydride (169mg, 0.80mmol) was added and the reaction stirred at room temperature for 16 hours. The reaction mixture was poured into aqueous sodium bicarbonate solution, and extracted with ethyl acetate. The combined organic extracts were dried ( $\text{MgSO}_4$ ) and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using dichloromethane:methanol:0.88 ammonia (91.75:7.5:0.75) as eluant to afford the title compound, 70mg;  $\delta$  ( $\text{CDCl}_3$ ) 1.02 (3H, t), 1.22 (3H, t), 1.58 (3H, t), 1.92 (2H, m), 2.14 (2H, m), 2.25-2.45 (7H, m), 2.54 (4H, m), 2.91 (2H, q), 2.99-3.16 (6H, m), 4.08 (1H, m), 4.78 (2H, q), 5.11 (1H, br s), 6.65 (1H, br s), 8.63 (1H, d), 8.83 (1H, d), 10.53 (1H, s).

### PDE5i Example 3

5-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-phenyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

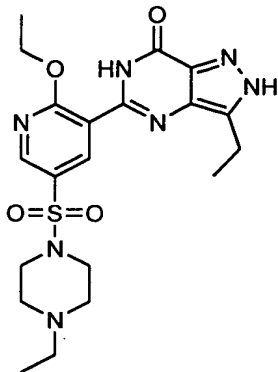


Pyridine (0.1ml, 1.08mmol) was added to a mixture of the product from stage a) below (250mg, 0.54mmol), copper (II) acetate monohydrate (145mg, 0.72mmol), benzenboronic acid (132mg, 1.08mmol) and 4Å molecular sieves (392mg) in dichloromethane (5ml), and the reaction stirred at room temperature for 4 days. The reaction mixture was filtered and the filtrate evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane:methanol:0.88 ammonia (97:3:0.5) as eluant, and triturated with ether:hexane. The resulting solid was filtered and recrystallised from *iso*-propanol:dichloromethane to give the title compound as a solid, 200mg,  $\delta$  ( $\text{CDCl}_3$ ) 1.02 (3H, t), 1.47 (3H, t), 1.60 (3H, t), 2.42 (2H, q), 2.58 (4H, m), 3.10 (2H, q), 3.17 (4H, m), 4.76 (2H, q), 7.40 (1H, m), 7.51 (2H, m), 7.80 (2H, d), 8.67 (1H, d), 9.16 (1H, s), 10.90 (1H, s); LRMS :  $m/z$  538 ( $M+1$ )<sup>+</sup>.



Preparation of Starting Materials for PDE5i Example 3

- a) 5-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one



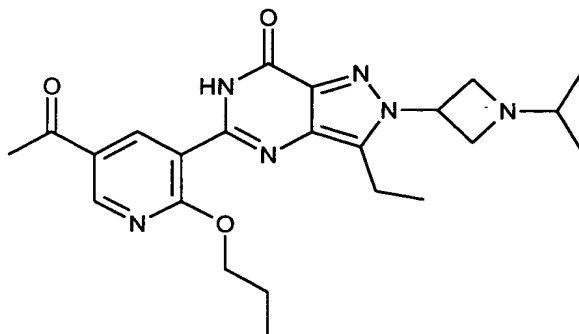
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Potassium bis(trimethylsilyl)amide (8.28g, 41.6mmol) was added to a solution of the product from Example 1, stage h) (10.0g, 20.8mmol) and ethyl acetate (2ml, 20mmol) in ethanol (160ml), and the reaction mixture heated at 120°C for 12 hours in a sealed vessel. The cooled mixture was evaporated under reduced pressure and the residue was purified by column chromatography on silica gel using dichloromethane:methanol:0.88 ammonia (95:5:0.5) as eluant, to give the title compound, 3.75g;  $\delta$  (CDCl<sub>3</sub>) 1.03 (3H, t), 1.42 (3H, t), 1.60 (3H, t), 2.42 (2H, q), 2.58 (4H, m), 3.02 (2H, q), 3.16 (4H, m), 4.78 (2H, q), 8.66 (1H, d), 9.08 (1H, d), 11.00 (1H, s) 11.05-11.20 (1H, br s), LRMS : m/z 462 (M+1)<sup>+</sup>.

15

PDE5i Example 4

- 5-(5-Acetyl-2-propoxy-3-pyridinyl)-3-ethyl-2-(1-isopropyl-3-azetidynyl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

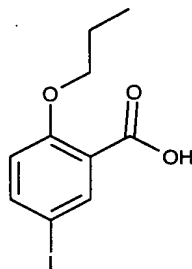


- 20 The product from stage h) below (0.23 mmol) was dissolved in dichloromethane (10 ml) and acetone (0.01 ml) was added. After 30 min stirring sodium triacetoxyborohydride (0.51 mmol) was added and stirring continued for 14 h.

Further acetone (0.01 ml) and sodium triacetoxyborohydride (0.51 mmol) were added and stirring continued for a further 4.5 h. Starting material still remained so further acetone (0.01 ml) and sodium triacetoxyborohydride (0.51 mmol) were added and stirring continued for a further 18 h. The reaction mixture was diluted with dichloromethane, washed with sodium bicarbonate solution then brine, dried (MgSO<sub>4</sub>) and concentrated. Purification by flash column chromatography (elution with 94:6:0.6 dichloromethane/methanol/0.88 ammonia) gave the product as a solid, M.p. 162.8-163.6°C; <sup>1</sup>H NMR (400MHz, MeOD): δ = 1.00 (app. d, 9H), 1.30 (t, 3H), 1.84 (app. q, 2H), 2.60 (s, 3H), 2.62-2.72 (m, 1H), 3.00-3.10 (q, 2H), 3.75 (t, 2H), 3.90 (t, 2H), 4.50 (t, 2H), 5.25 (t, 1H), 8.70 (s, 1H), 8.90 (s, 1H); LRMS (TSP – positive ion) 439 (MH<sup>+</sup>); Anal. Found C, 61.92; H, 6.84; N, 18.70 Calcd for C<sub>23</sub>H<sub>30</sub>O<sub>3</sub>N<sub>6</sub>·0.1CH<sub>2</sub>Cl<sub>2</sub>: C, 62.07; H, 6.81; N, 18.80.

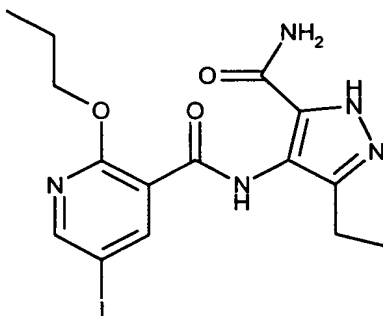
#### Preparation of Starting Materials for PDE5i Example 4

##### a) 2-Propoxy-5-iodonicotinic acid



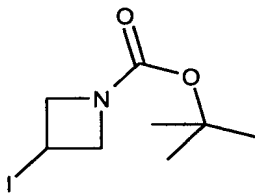
*N*-Iodosuccinamide (18.22 g, 0.08 mol), trifluoroacetic acid (100 ml) and trifluoroacetic anhydride (25 ml) were added to 2-propoxynicotinic acid (0.054 mol). The mixture was refluxed for 2.5 h, cooled and the solvents evaporated. The residue was extracted from water with ethyl acetate and the organics washed with water (twice) and brine (twice), dried (MgSO<sub>4</sub>) and concentrated. The red residue was redissolved in ethyl acetate washed with sodium thiosulfate solution (twice), water (twice), brine (twice), redried (MgSO<sub>4</sub>) and concentrated to give the desired product as a solid; <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ = 1.05 (t, 3H), 1.85-2.0 (m, 2H), 4.5 (t, 2H), 8.5 (s, 1H), 8.6 (s, 1H); Analysis: found C, 35.16; H, 3.19; N, 4.46. Calcd for C<sub>9</sub>H<sub>10</sub>INO<sub>3</sub>: C, 35.19; H, 3.28; N, 4.56%.

##### b) *N*-[3-(Aminocarbonyl)-5-ethyl-1*H*-pyrazol-4-yl]-5-iodo-2-propoxy-nicotinamide



Oxalyl chloride (15.9 mmol) was added to a stirred solution of the product from stage a) (3.98 mmol) in dichloromethane (20 ml) and 3 drops *N,N*-dimethylformamide added. After 2.5 h the solvent was evaporated and the residue azeotroped 3 times with dichloromethane. The residue was resuspended in dichloromethane (4 ml) and added to a stirred mixture 4-amino-3-ethyl-1*H*-pyrazole-5-carboxamide (prepared as described in WO 98/49166) (3.58 mmol) and triethylamine (7.97 mmol) in dichloromethane (10 ml). After 1 h the solvent was evaporated and the residue partitioned between ethyl acetate and water. The organic phase was separated and washed with 2N HCl (twice), sodium bicarbonate solution (twice) and brine before being dried (MgSO<sub>4</sub>) and concentrated. The product was triturated with ether and filtered to give the title product as a solid. The mother liquor was concentrated and purified by flash column chromatography (elution with 80% ethyl acetate : hexane) to give further product; <sup>1</sup>H NMR (300 MHz, d<sub>4</sub>-MeOH): δ = 1.0 (t, 3H), 1.25 (t, 3H), 1.85-2.0 (m, 2H), 2.8 (q, 2H), 4.5 (t, 2H), 8.5 (s, 1H), 8.6 (s, 1H); LRMS (TSP) 444 (MH<sup>+</sup>).

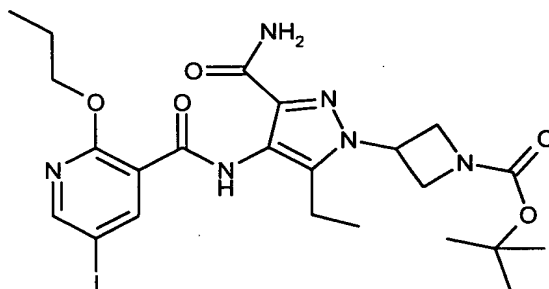
c) *tert*-Butyl 3-iodo-1-azetidinecarboxylate



A mixture of *tert*-butyl 3-[(methylsulfonyl)oxy]-1-azetidinecarboxylate (prepared as described in *Synlett* 1998, 379; 5.0 g, 19.9 mmol), and potassium iodide (16.5 g, 99.4 mmol) in *N,N*-dimethylformamide (25 ml), was heated at 100°C for 42 h. The cooled mixture was partitioned between water and ethyl acetate, and the layers separated. The organic phase was dried over MgSO<sub>4</sub>, concentrated under reduced pressure and the residue azeotroped with xylene. The crude product was purified by flash column chromatography (dichloromethane as eluant) to give the title compound, 3.26 g; <sup>1</sup>H

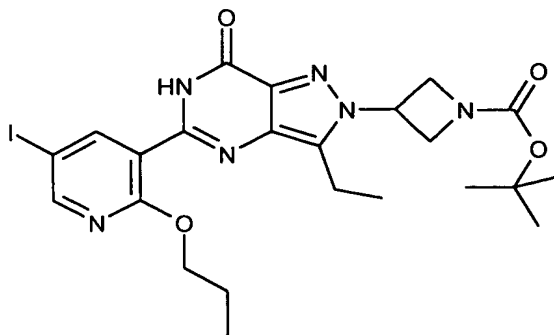
NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  = 1.43 (s, 9H), 4.28 (m, 2H), 4.46 (m, 1H), 4.62 (m, 2H); LRMS (TSP) 284 (MH)<sup>+</sup>

d) *tert*-Butyl 3-(3-(aminocarbonyl)-5-ethyl-4-[(5-iodo-2-propoxy-3-pyridinyl)carbonyl]amino}-1*H*-pyrazol-1-yl)-1-azetidinecarboxylate



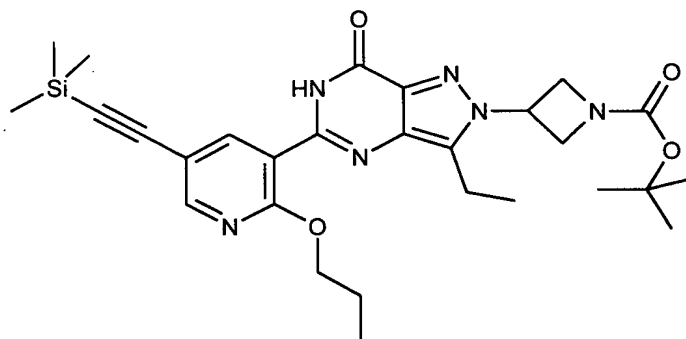
Cesium carbonate (3.59 mmol) was added to a stirred solution of the product from stage b) (1.79 mmol) and the product from stage c) (2.15 mmol) in *N,N*-dimethylformamide (10 ml) under a nitrogen atmosphere. The mixture was heated at 80°C for 24 h. The mixture was cooled and extracted from water with ethyl acetate. The organics were dried (MgSO<sub>4</sub>) and concentrated to give a brown oil. Purification by flash column chromatography (gradient elution from 100% dichloromethane to 90% dichloromethane/MeOH) gave the title product; <sup>1</sup>H NMR (400MHz, DMSO):  $\delta$  = 0.95 (t, 3H), 1.05 (t, 3H), 1.40 (s, 9H), 1.78-1.88 (m, 2H), 2.68 (q, 2H), 4.22-4.35 (m, 4H), 4.40 (t, 2H), 5.33 (t, 1H), 7.35 (bs, 1H), 7.52 (bs, 1H), 8.40 (s, 1H), 8.55 (s, 1H), 10.10 (s, 1H); LRMS (TSP – positive ion) 373.2 (MH<sup>+</sup> - BOC and I); Anal. Found C, 45.11; H, 5.07; N, 13.56 Calcd for C<sub>23</sub>H<sub>31</sub>O<sub>5</sub>N<sub>6</sub>I. 0.2 DCM: C, 45.28; H, 5.14; N, 13.66.

e) *tert*-Butyl 3-[3-ethyl-5-(5-iodo-2-propoxy-3-pyridinyl)-7-oxo-6,7-dihydro-2*H*-pyrazolo[4,3-*d*]pyrimidin-2-yl]-1-azetidinecarboxylate



The product from stage d) (28.4 mmol) was dissolved in n-propanol (200 ml), ethyl acetate (6 ml) and potassium t-butoxide (28.4 mmol) were added and the resultant mixture heated to reflux for 6h. Additional potassium t-butoxide (14.2 mmol) was added and the mixture heated for a further 2h, after which the solvent was removed *in vacuo*. The residue was partitioned between water (50 ml) and methylene chloride (100 ml) and the organic phase separated. The aqueous phase was extracted with dichloromethane (2 x 100 ml) and the combined organics dried over MgSO<sub>4</sub> and reduced to a solid. Purification by column chromatography (elution with ethyl acetate) gave the title compound; <sup>1</sup>H NMR (400MHz, CDCl<sub>3</sub>): δ = 1.05 (t, 3H), 1.30 (t, 3H), 1.43 (s, 9H), 1.87-1.96 (m, 2H), 3.00 (q, 2H), 4.34 (t, 2H), 4.49 (t, 2H), 4.60 (br s, 2H), 5.20 (t, 1H), 8.41 (d, 1H), 8.94 (s, 1H), 10.75 (br s, 1H); LRMS (TSP – positive ion) 598.1 (MNH<sub>4</sub><sup>+</sup>); Anal. Found C, 47.54; H, 5.02; N, 14.09 Calcd for C<sub>23</sub>H<sub>29</sub>O<sub>4</sub>N<sub>6</sub>: C, 47.60; H, 5.04; N, 14.48.

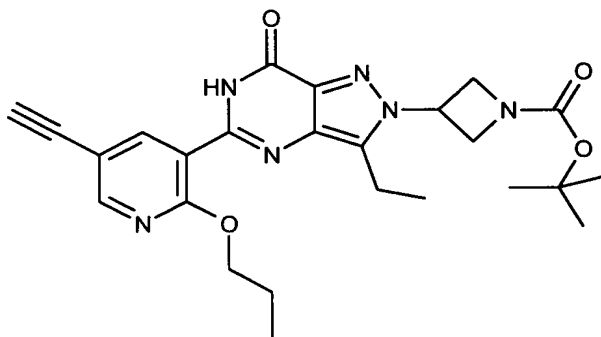
- f) *tert*-Butyl 3-(3-ethyl-7-oxo-5-{2-propoxy-5-[(trimethylsilyl)ethynyl]-3-pyridinyl}-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)-1-azetidinecarboxylate



The product from stage e) (0.25 mmol) was suspended in triethylamine (2 ml) and trimethylsilylacetylene (0.39 mmol) and acetonitrile (2 ml to try and solubilise reactants). Pd(PPh<sub>3</sub>)<sub>2</sub>Cl<sub>2</sub> (0.006 mmol) and cuprous iodide (0.006 mmol) were added and the reaction mixture stirred. After 1 h a further portion of trimethylsilylacetylene (0.19 mmol) was added and stirring continued for 2 h. The solvent was evaporated and the residue partitioned between ethyl acetate and water. The organics were washed with brine, dried (MgSO<sub>4</sub>) and concentrated. Purification by flash column chromatography (gradient elution from 100% dichloromethane to 99% dichloromethane/methanol) gave the title compound; <sup>1</sup>H NMR (400MHz, MeOD): δ = 0.25 (s, 9H), 1.05 (t, 3H), 1.31 (t, 3H), 1.44 (s, 9H), 1.87-1.96 (m, 2H), 3.00 (q, 2H), 4.33 (t, 2H), 4.52 (t, 2H), 4.54-4.80 (m, 2H), 5.18-5.25 (m, 1H), 8.32 (d, 1H), 8.74 (d,

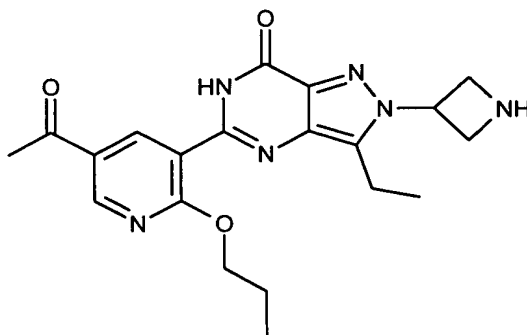
1H); LRMS (TSP – positive ion) 569 (MNH<sub>4</sub><sup>+</sup>), 452.0 (MH<sup>+</sup>); Anal. Found C, 60.82; H, 6.90; N, 15.15 Calcd for C<sub>28</sub>H<sub>38</sub>O<sub>4</sub>N<sub>6</sub>Si: C, 61.07; H, 6.95; N, 15.26.

- g) tert-Butyl 3-[3-ethyl-5-(5-ethynyl-2-propoxy-3-pyridinyl)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl]-1-azetidinecarboxylate



Potassium fluoride (0.38 mmol) was added to a stirred solution of the product of stage f) (0.19 mmol) in aqueous *N,N*-dimethylformamide (2 ml *N,N*-dimethylformamide /0.2 ml water) at 0°C. After 10 min the reaction was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was diluted with ethyl acetate and washed with water, 1 *N* hydrochloric acid (3 times) and brine. The organic layer was dried (MgSO<sub>4</sub>) and concentrated to give the title compound as a solid; 1H NMR (400MHz, CDCl<sub>3</sub>): δ = 1.05 (t, 3H), 1.30 (t, 3H), 1.43 (s, 9H), 1.88-2.00 (m, 2H), 3.00 (q, 2H), 3.19 (s, 1H), 4.35 (app t, 2H), 4.52 (app t, 2H), 4.60-4.80 (br s, 2H), 5.22 (t, 1H), 8.39 (s, 1H), 8.80 (s, 1H), 10.75 (br s, 1H); LRMS (TSP – positive ion) 496 (MNH<sub>4</sub><sup>+</sup>).

- h) 5-(5-Acetyl-2-propoxy-3-pyridinyl)-2-(3-azetidiny)-3-ethyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one



The product from stage g) (1.44 g, 3.0 mmol) in acetone (50 ml) and sulphuric acid (1N, 3 ml) was treated with mercuric sulphate (268 mg, 9.0 mmol) and heated to

reflux for 6h. The reaction mixture was concentrated to ~20 ml *in vacuo*, poured into sodium bicarbonate (sat. aq., 20ml) and extracted into methylene chloride (6 x 20 ml). Combined organics were washed with brine (20 ml), dried over MgSO<sub>4</sub>, and concentrated to a brown oil which was taken up in 40% trifluoroacetic acid in methylene chloride (50ml) and water (1 ml) and stirred for 1h at room temperature. After evaporation *in vacuo*, the residue was purified by column chromatography (eluting with 95:5:1 methylene chloride:methanol:0.88 ammonia) to afford the title compound as a white hygroscopic foam ( 1.65 g); m.p. 128.5-130.0°C; <sup>1</sup>H NMR (400MHz, MeOD):  $\delta$  = 1.00 (t, 3H), 1.30 (t, 3H), 1.79-1.90 (m, 2H), 2.60 (s, 3H), 3.00-3.10 (q, 2H), 4.50 (t, 2H), 4.60-4.70 (m, 4H), 5.65-5.78 (m, 1H), 8.65 (s, 1H), 8.90 (s, 1H); LRMS (TSP – positive ion) 397 (MH<sup>+</sup>).

#### PDE5i Example 5

##### 5-(5-acetyl-2-butoxy-3-pyridinyl)-3-ethyl-2-(1-ethyl-3-azetidiny)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one.

The title compound from preparation 5(a) (120 mg, 0.28 mmol) and cesium carbonate (274 mg, 0.84 mmol) were dissolved in *n*-butanol (4 ml), and heated at 90°C under nitrogen with molecular sieves for 96h. The mixture was then partitioned between water (10 ml) and dichloromethane (10 ml). The organic layer was separated, and the aqueous layer extracted further with dichloromethane (3 x 15 ml). The combined organic layers were dried (MgSO<sub>4</sub>), and concentrated *in vacuo*. The crude product was purified by flash column chromatography (95:5:0.5-90:10:1 ethyl acetate:methanol:0.88 NH<sub>3</sub> as eluents), to yield the title compound as a colourless glass (77 mg, 0.18 mmol).

**m.p.** 91.6-93.7°C

**<sup>1</sup>H NMR** (400MHz, CDCl<sub>3</sub>):  $\delta$  = 1.00-1.05 (m, 6H), 1.38 (t, 3H), 1.50-1.62 (m, 2H), 1.90-2.00 (m, 2H), 2.63 (s, 3H), 2.63-2.70 (m, 2H), 3.02 (q, 2H), 3.75 (t, 2H), 3.90 (t, 2H), 4.68 (t, 2H), 5.10-5.20 (m, 1H), 8.84 (s, 1H), 9.23 (s, 1H), 10.63 (br s, 1H).

**LRMS** (TSP – positive ion) 439 (MH<sup>+</sup>)

**Anal.** Found C, 60.73; H, 7.06; N, 18.03 Calcd for C<sub>23</sub>H<sub>30</sub>O<sub>3</sub>N<sub>6</sub>·0.2MeOH·0.1 DIPE: C, 60.88; H, 7.26; N, 17.90

#### Preparation of starting materials for Example 5

##### 5(a) 5-(5-Acetyl-2-propoxy-3-pyridinyl)-3-ethyl-2-(1-ethyl-3-azetidiny)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

Sodium cyanoborohydride (92 mg, 1.47 mmol) was added to a stirring solution of title compound from example 5(b) (500 mg, 0.98 mmol), acetaldehyde (64  $\mu$ l, 1.18 mmol) and sodium acetate (161 mg, 1.96 mmol) in methanol (10 ml) under nitrogen at room temperature. After 1h the mixture was poured into NaHCO<sub>3</sub> (sat. aq., 20 ml), and  
5 extracted with dichloromethane (3 x 15 ml). The combined organic layers were dried (MgSO<sub>4</sub>) and concentrated *in vacuo*. The crude product was purified by flash column chromatography (95:5:0.5-80:20:1 ethyl acetate:methanol:0.88 NH<sub>3</sub> as eluent) to yield the title compound as a white solid (140 mg, 0.33 mmol).

**<sup>1</sup>H NMR** (400MHz, CDCl<sub>3</sub>):  $\delta$  = 0.97 (t, 3H), 1.03 (t, 3H), 1.30 (t, 3H), 2.82-2.97 (m, 2H), 2.58-2.65 (m, 5H), 2.98 (q, 2H), 3.68 (t, 2H), 3.85 (dd, 2H), 4.58 (dd, 2H), 5.05-5.17 (m, 1H), 8.79 (s, 1H), 9.18 (s, 1H), 10.62 (br s, 1H).

**LRMS** (TSP – positive ion) 426 (MH<sup>+</sup>)

5(b) 5-(5-Acetyl-2-propoxy-3-pyridinyl)-2-(3-azetidiny)-3-ethyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

The title compound of Preparation 5(c) (1.44 g, 3.0 mmol) in acetone (50 ml) and sulphuric acid (1N, 3 ml) was treated with mercuric sulphate (268 mg, 9.0 mmol) and heated to reflux for 6h. The reaction mixture was concentrated to ~20 ml *in vacuo*, poured into sodium bicarbonate (sat. aq., 20ml) and extracted into methylene chloride (6 x 20 ml). Combined organics were washed with brine (20 ml), dried over  
20 MgSO<sub>4</sub>, and concentrated to a brown oil which was taken up in 40% trifluoroacetic acid in methylene chloride (50ml) and water (1 ml) and stirred for 1h at room temperature. After evaporation *in vacuo*, the residue was purified by column chromatography (eluting with 95:5:1 methylene chloride:methanol:0.88 ammonia) to afford the title compound as a white hygroscopic foam ( 1.65 g).

**m.p.** 128.5-130.0°C

**<sup>1</sup>H NMR** (400MHz, MeOD):  $\delta$  = 1.00 (t, 3H), 1.30 (t, 3H), 1.79-1.90 (m, 2H), 2.60 (s, 3H), 3.00-3.10 (q, 2H), 4.50 (t, 2H), 4.60-4.70 (m, 4H), 5.65-5.78 (m, 1H), 8.65 (s, 1H), 8.90 (s, 1H)

**LRMS** (TSP – positive ion) 397 (MH<sup>+</sup>)

5(c) tert-Butyl 3-[3-ethyl-5-(5-ethynyl-2-propoxy-3-pyridinyl)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl]-1-azetidinecarboxylate

Prepared from the title compound of Preparation 5(d) by the method of Preparation  
35 5(c)(i).



**<sup>1</sup>H NMR** (400MHz, CDCl<sub>3</sub>): δ = 1.05 (t, 3H), 1.30 (t, 3H), 1.43 (s, 9H), 1.88-2.00 (m, 2H), 3.00 (q, 2H), 3.19 (s, 1H), 4.35 (app t, 2H), 4.52 (app t, 2H), 4.60-4.80 (br s, 2H), 5.22 (t, 1H), 8.39 (s, 1H), 8.80 (s, 1H), 10.75 (br s, 1H)

**LRMS** (TSP – positive ion) 496 (MNH<sub>4</sub><sup>+</sup>).

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5(c)(i) 5-(2-Butoxy-5-ethynyl-3-pyridinyl)-3-ethyl-2-(2-methoxyethyl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

Potassium fluoride (22 mg, 0.38 mmol) was added to a stirred solution of the title compound of Preparation 5(d)(i) (90 mg, 0.19 mmol) in aqueous *N,N*-dimethylformamide (2 mL *N,N*-dimethylformamide /0.2 mL water) at 0°C. After 10 min the reaction was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was diluted with ethyl acetate and washed with water, 1 *N* hydrochloric acid (3 times) and brine. The organic layer was dried (MgSO<sub>4</sub>) and concentrated to give the title compound as a white solid (75 mg).

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**<sup>1</sup>H NMR** (400 MHz, CDCl<sub>3</sub>): δ = 1.00 (t, 3H), 1.40 (t, 3H), 1.50 (m, 2H), 1.90 (m, 2H), 3.05 (q, 2H), 3.20 (s, 1H), 3.30 (s, 3H), 3.85 (t, 2H), 4.40 (t, 2H), 4.60 (t, 2H), 8.40 (s, 1H), 8.80 (s, 1H), 10.70 (s, 1H).

**LRMS** (TSP): 396.3 (MH<sup>+</sup>).

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4(d) *tert*-Butyl 3-(3-ethyl-7-oxo-5-{2-propoxy-5-[(trimethylsilyl)ethynyl]-3-pyridinyl}-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)-1-azetidinecarboxylate

Prepared from the title compound of Preparation 5(e) by the method of Preparation 5(d)(i).

**<sup>1</sup>H NMR** (400MHz, MeOD): δ = 0.25 (s, 9H), 1.05 (t, 3H), 1.31 (t, 3H), 1.44 (s, 9H), 1.87-1.96 (m, 2H), 3.00 (q, 2H), 4.33 (t, 2H), 4.52 (t, 2H), 4.54-4.80 (m, 2H), 5.18-5.25 (m, 1H), 8.32 (d, 1H), 8.74 (d, 1H)

**LRMS** (TSP – positive ion) 569 (MNH<sub>4</sub><sup>+</sup>), 552.0 (MH<sup>+</sup>)

**Anal.** Found C, 60.82; H, 6.90; N, 15.15 Calcd for C<sub>28</sub>H<sub>38</sub>O<sub>4</sub>N<sub>6</sub>Si: C, 61.07; H, 6.95; N, 15.26.

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5(d)(i) 5-(2-Butoxy-5-trimethylsilyl-ethynyl-3-pyridinyl)-3-ethyl-2-(2-methoxy-ethyl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

The title compound from Example 1 of PCT application IB00/1430 (127 mg, 0.25 mmol) was suspended in triethylamine (2 mL) and trimethylsilylacetylene (38 mg, 0.39 mmol) and acetonitrile (2 mL). Pd(PPh<sub>3</sub>)<sub>2</sub>Cl<sub>2</sub> (5 mg, 0.006 mmol) and cuprous

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iodide (1.2 mg, 0.006 mmol) were added and the reaction mixture stirred. After 1 h a further portion of trimethylsilylacetylene (19 mg, 0.19 mmol) was added and stirring continued for 2 h. The solvent was evaporated and the residue partitioned between ethyl acetate and water. The organics were washed with brine, dried (MgSO<sub>4</sub>) and concentrated to give a brown foam. Purification by flash column chromatography (gradient elution from 100% dichloromethane to 99% dichloromethane/methanol) gave the title compound as a light brown solid (108 mg).

**<sup>1</sup>H NMR** (300 MHz, CDCl<sub>3</sub>):  $\delta$  = 0.25 (s, 9H), 1.00 (t, 3H), 1.40 (t, 3H), 1.50 (m, 2H), 1.90 (m, 2H), 3.10 (q, 2H), 3.30 (s, 3H), 3.90 (t, 2H), 4.40 (t, 2H), 4.60 (t, 2H), 8.40 (s, 1H), 8.80 (s, 1H), 10.70 (s, 1H).

**LRMS** (TSP): 468.3 (MH<sup>+</sup>).

5(e) *tert*-Butyl 3-[3-ethyl-5-(5-iodo-2-propoxy-3-pyridinyl)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl]-1-azetidinecarboxylate

The title compound was prepared from the product of Preparation 5(f) using the method of Preparation 5(e)(i).

**<sup>1</sup>H NMR** (400MHz, CDCl<sub>3</sub>):  $\delta$  = 1.05 (t, 3H), 1.30 (t, 3H), 1.43 (s, 9H), 1.87-1.96 (m, 2H), 3.00 (q, 2H), 4.34 (t, 2H), 4.49 (t, 2H), 4.60 (br s, 2H), 5.20 (t, 1H), 8.41 (d, 1H), 8.94 (s, 1H), 10.75 (br s, 1H)

**LRMS** (TSP – positive ion) 598.1 (MNH<sub>4</sub><sup>+</sup>)

**Anal.** Found C, 47.54; H, 5.02; N, 14.09 Calcd for C<sub>23</sub>H<sub>29</sub>O<sub>4</sub>N<sub>6</sub>I: C, 47.60; H, 5.04; N, 14.48.

5(e)(i) 3-Ethyl-5-(5-iodo-2-propoxy-3-pyridinyl)-1-[2-(4-morpholinyl)ethyl]-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

The title compound of Preparation 48 of PCT application IB00/1430 (15.78 g, 28.4 mmol) was dissolved in n-propanol (200 ml), ethyl acetate (6 ml) and potassium t-butoxide (3.2 g, 28.4 mmol) were added and the resultant mixture heated to reflux for 6h. Additional potassium t-butoxide (1.6 g, 14.2 mmol) was added and the mixture heated for a further 2h, after which the solvent was removed *in vacuo*. The residue was partitioned between water (50 ml) and methylene chloride (100 ml) and the organic phase separated. The aqueous phase was extracted with dichloromethane (2 x 100 ml) and the combined organics dried over MgSO<sub>4</sub> and reduced to a yellow solid (~17 g). Purification by column chromatography (elution with ethyl acetate) gave the title compound (13.3 g, 24.1 mmol) together with recovered starting material (2.31 g, 4.2 mmol).

m.p. 175-177°C.

**<sup>1</sup>H NMR** (300 MHz, CDCl<sub>3</sub>): δ = 1.1 (t, 3H), 1.4 (t, 3H), 1.9-2.05 (m, 2H), 2.45-2.55 (m, 4H), 2.85 (t, 2H), 3.0 (q, 2H), 3.6-3.65 (m, 4H), 4.5 (t, 2H), 4.7 (t, 2H), 8.4 (s, 1H), 9.0 (s, 1H), 10.95 (br s, 1H).

5 **LRMS** (TSP) 540 (MH<sup>+</sup>).

**Analysis:** found C, 46.79; H, 5.01; N, 15.44. Calcd for C<sub>21</sub>H<sub>27</sub>N<sub>6</sub>O<sub>3</sub>I : C, 46.85; H, 5.05; N, 15.61%

10 5(f) *tert*-Butyl 3-(3-(aminocarbonyl)-5-ethyl-4-[(5-iodo-2-propoxy-3-pyridinyl)carbonyl]amino)-1*H*-pyrazol-1-yl)-1-azetidinecarboxylate

The title compound was prepared by the method of Preparation 5(f)(i) using the products from Preparations 5(g) and 5(i).

15 **<sup>1</sup>H NMR** (400MHz, DMSO): δ = 0.95 (t, 3H), 1.05 (t, 3H), 1.40 (s, 9H), 1.78-1.88 (m, 2H), 2.68 (q, 2H), 4.22-4.35 (m, 4H), 4.40 (t, 2H), 5.33 (t, 1H), 7.35 (bs, 1H), 7.52 (bs, 1H), 8.40 (s, 1H), 8.55 (s, 1H), 10.10 (s, 1H)

**LRMS** (TSP – positive ion) 373.2 (MH<sup>+</sup> - BOC and I)

**Anal.** Found C, 45.11; H, 5.07; N, 13.56 Calcd for C<sub>23</sub>H<sub>31</sub>O<sub>5</sub>N<sub>6</sub>I. 0.2 DCM: C, 45.28; H, 5.14; N, 13.66.

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5(f)(i) *N*-{3-(Aminocarbonyl)-1-[2-dimethylamino)ethyl]-5-ethyl-1*H*-pyrazol-4-yl}-2-butoxy-5-iodonicotinamide

Cesium carbonate (1.17 g, 3.59 mmol) was added to a stirred solution of the title compound from Preparation 16 of PCT application IB00/1430 (800 mg, 1.79 mmol) and *N,N*-dimethylaminoethyl chloride hydrochloride (309 mg, 2.15 mmol) in *N,N*-dimethylformamide (10 mL) under a nitrogen atmosphere. The mixture was heated at 80°C for 24 h. The mixture was cooled and extracted from water with ethyl acetate. The organics were dried (MgSO<sub>4</sub>) and concentrated to give a brown oil. Purification by flash column chromatography (gradient elution from 100% dichloromethane to 90% dichloromethane/MeOH) gave the product as a pale brown oil (522 mg).

30 **<sup>1</sup>H NMR** (400 MHz, CDCl<sub>3</sub>): δ = 0.95 (t, 3H), 1.20 (t, 3H), 1.40 (m, 2H), 1.90 (m, 2H), 2.35 (s, 6H), 2.80 (t, 2H), 2.85 (q, 2H), 4.20 (t, 2H), 4.60 (t, 2H), 5.30 (br s, 1H), 6.60 (br s, 1H), 8.40 (s, 1H), 8.75 (s, 1H), 10.35 (s, 1H).

35 **LRMS** (TSP): 529.5 (MH<sup>+</sup>).

5(g) *N*-[3-(Aminocarbonyl)-5-ethyl-1*H*-pyrazol-4-yl]-5-iodo-2-propoxy-nicotinamide

The title compound was prepared from 2-propoxy-5-iodonicotinic acid (see Preparation 5(h) and 4-amino-3-ethyl-1*H*-pyrazole-5-carboxamide (prepared as described in WO 98/49166) according to the method described in Preparation 5(g)(i).

**<sup>1</sup>H NMR** (300 MHz, d<sub>4</sub>-MeOH): δ = 1.0 (t, 3H), 1.25 (t, 3H), 1.85-2.0 (m, 2H), 2.8 (q, 2H), 4.5 (t, 2H), 8.5 (s, 1H), 8.6 (s, 1H).

**LRMS** (TSP) 444 (MH<sup>+</sup>).

5(g)(i) *N*-[3-(Aminocarbonyl)-5-ethyl-1-(2-methoxyethyl)-1*H*-pyrazol-4-yl]-2-butoxy-5-iodonicotinamide

Oxalyl chloride (2 g, 15.9 mmol) was added to a stirred solution of the title compound from Preparation 4 of PCT application IB00/1430 (1.28 g, 3.98 mmol) in dichloromethane (20 mL) and 3 drops *N,N*-dimethylformamide added. After 2.5 h the solvent was evaporated and the residue azeotroped 3 times with dichloromethane.

The residue was resuspended in dichloromethane (4 mL) and added to a stirred mixture of the title compound of Preparation 11 from PCT application IB00/1430 (0.76 g, 3.58 mmol) and triethylamine (0.8 g, 7.97 mmol) in dichloromethane (10 mL). After 1 h the solvent was evaporated and the residue partitioned between ethyl acetate and water. The organic phase was separated and washed with 2N HCl (twice), sodium bicarbonate solution (twice) and brine before being dried (MgSO<sub>4</sub>) and concentrated. The product was triturated with ether and filtered to give 820 mg of pure product as a white solid. The mother liquor was concentrated and purified by flash column chromatography (elution with 80% ethyl acetate : hexane), to give a further 605 mg of product.

**<sup>1</sup>H NMR** (400 MHz, CDCl<sub>3</sub>): δ = 0.95 (t, 3H), 1.20 (t, 3H), 1.45 (m, 2H), 1.90 (m, 2H), 2.85 (q, 2H), 3.35 (s, 3H), 3.80 (t, 2H), 4.25 (t, 2H), 4.60 (t, 2H), 5.20 (br s, 1H), 6.60 (br s, 1H), 8.40 (s, 1H), 8.80 (s, 1H), 10.30 (s, 1H).

**LRMS** (TSP): 516.2 (MH<sup>+</sup>).

5(h) 2-Propoxy-5-iodonicotinic acid

The title compound was prepared from 2-propoxy nicotinic acid (prepared as described in WO 99/54333, the compound 2-*n*-propoxypyridine-3-carboxylic acid, Preparation 46 prepared by the process of Preparation 1) using the method of Preparation 5(h)(i).

**<sup>1</sup>H NMR** (300 MHz, CDCl<sub>3</sub>): δ = 1.05 (t, 3H), 1.85-2.0 (m, 2H), 4.5 (t, 2H), 8.5 (s, 1H), 8.6 (s, 1H).

**Analysis:** found C, 35.16; H, 3.19; N, 4.46. Calcd for  $C_9H_{10}INO_3$ : C, 35.19; H, 3.28; N, 4.56%

5(h)(i) 2-isoButoxy-5-iodo nicotinic acid

5 *N*-Iodosuccinamide (18.22 g, 0.08 mol), trifluoroacetic acid (100 mL) and trifluoroacetic anhydride (25 mL) were added to 2-isobutoxynicotinic acid (10.55 g, 0.054 mol). The mixture was refluxed for 2.5 h, cooled and the solvents evaporated. The residue was extracted from water with ethyl acetate and the organics washed with water (twice) and brine (twice), dried ( $MgSO_4$ ) and concentrated. The red  
10 residue was redissolved in ethyl acetate washed with sodium thiosulfate solution (twice), water (twice), brine (twice), redried ( $MgSO_4$ ) and concentrated to give the desired product as a yellow solid.

$^1H$  NMR (300 MHz,  $CDCl_3$ ):  $\delta$  = 1.05 (d, 6H), 2.20 (m, 1H), 4.40 (d, 2H), 8.50 (s, 1H), 8.70 (s, 1H),

15 LRMS (TSP): 322.3 ( $MH^+$ ).

5(i) *tert*-Butyl 3-iodo-1-azetidinecarboxylate

A mixture of *tert*-butyl 3-[(methylsulfonyl)oxy]-1-azetidinecarboxylate (prepared as described in *Synlett* **1998**, 379; 5.0 g, 19.9 mmol), and potassium iodide (16.5 g, 99.4  
20 mmol) in *N,N*-dimethylformamide (25 mL), was heated at 100°C for 42 h. The cooled mixture was partitioned between water and ethyl acetate, and the layers separated. The organic phase was dried over  $MgSO_4$ , concentrated under reduced pressure and the residue azeotroped with xylene. The crude product was purified by flash column chromatography (dichloromethane as eluant) to give the title compound, 3.26 g.

25  $^1H$  NMR (300 MHz,  $CDCl_3$ )  $\delta$  = 1.43 (s, 9H), 4.28 (m, 2H), 4.46 (m, 1H), 4.62 (m, 2H).  
LRMS (TSP) 284 ( $MH^+$ )

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and  
30 system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes  
35 for carrying out the invention which are obvious to those skilled in biochemistry and

biotechnology or related fields are intended to be within the scope of the following claims.

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## 15 ABBREVIATIONS

cAMP = cyclic adenosine-3',5'-monophosphate

cGMP = cyclic guanosine-3',5'-monophosphate

20 P<sub>cGMP</sub> = potentiator of cGMP

NEP = neutral endopeptidase

NEPi = inhibitor of NEP (also known as I:NEP)

15 VIP = vasoactive intestinal peptide

PDE = phosphodiesterase

PDE<sub>n</sub> = PDE family (e.g. PDE1, PDE2 etc.)

PDE<sub>cGMP</sub> = cGMP hydrolysing PDE

30 PDEi = inhibitor of a PDE (also known as I:PDE)

NPY = neuropeptide Y

I:NPY = inhibitor of NPY

35 kDa = kilodalton

bp = base pair

kb = kilobase pair

Claims

1. A pharmaceutical composition for use (or when in use) in the treatment of MED; the pharmaceutical composition comprising an agent capable of potentiating cAMP in the sexual genitalia of a male suffering from MED; wherein the agent is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient; and wherein said agent is an inhibitor of NPY (I:NPY).
2. A pharmaceutical composition according to claim 1 wherein the agent is a mediator of genital vasorelaxation.
3. A pharmaceutical composition according to claim 1 or claim 2 wherein the composition is for oral administration.
4. A pharmaceutical composition according to any one of claims 1 to 3 wherein the said cAMP is endogenous cAMP.
5. A pharmaceutical composition according to any one of claims 1 to 4 wherein the composition is applied before or during sexual stimulation.
6. Use of an agent in the manufacture of a medicament for the treatment of MED; wherein the agent is capable of potentiating cAMP in the sexual genitalia of a male suffering from MED; and wherein said agent is an I:NPY.
7. Use according to claim 6 wherein the agent is a mediator of genital vasorelaxation.
8. Use according to claim 6 or claim 7 wherein the medicament is for oral administration.
9. Use according to any one of claims 6 to 8 wherein the said cAMP is endogenous cAMP.
10. Use according to any one of claims 6 to 9 wherein the composition is applied before or during sexual stimulation.

11. A method of treating a male (such as a male suffering from MED); the method comprising delivering to the male an agent that is capable of potentiating cAMP in the sexual genitalia; wherein the agent is in an amount to cause potentiation of cAMP in the sexual genitalia of the male; wherein the agent is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient; and wherein said agent is an I:NPY.
12. A method according to claim 11 wherein the agent is a mediator of genital vasorelaxation.
13. A method according to claim 11 or claim 12 wherein the agent is orally administered.
14. A method according to any one of claims 11 to 13 wherein the said cAMP is endogenous cAMP.
15. A pharmaceutical composition according to any one of claims 11 to 14 wherein the composition is applied before or during sexual stimulation.
16. An assay method for identifying an agent that can be used to treat MED, the assay method comprising: determining whether an agent can directly or indirectly potentiate cAMP; wherein a potentiation of cAMP in the presence of the agent is indicative that the agent may be useful in the treatment of MED; and wherein said agent is an I:NPY.
17. A process comprising the steps of:
- (a) performing the assay according to claim 16;
  - (b) identifying one or more agents that can directly or indirectly potentiate cAMP; and
  - (c) preparing a quantity of those one or more identified agents;
- and wherein said agent is an I:NPY.
18. A method of treating MED, by potentiating *in vivo* cAMP with an agent;

wherein the agent is capable of directly or indirectly potentiating cAMP in an *in vitro* assay method;

5 wherein the *in vitro* assay method is the assay method defined in claim 16;

and wherein said agent is an I:NPY.

10 19. Use of an agent in the preparation of a pharmaceutical composition for the treatment of MED, wherein the agent is capable of directly or indirectly potentiating cAMP when assayed *in vitro* by the assay method according to claim 16; and wherein said agent is an I:NPY.

15 20. An agent identified by the assay method according to claim 16; and wherein said agent is an I:NPY.

21. An agent according to claim 20 for use in medicine; and wherein said agent is an I:NPY.

20 22. An agent according to claim 21 for use in treating MED; and wherein said agent is an I:NPY.

25 23. A medicament for oral administration to treat MED, wherein the medicament comprises the agent according to claim 20; and wherein said agent is an I:NPY.

30 24. A diagnostic method, the method comprising isolating a sample from a male; determining whether the sample contains an entity present in such an amount to cause MED, or is in an amount so as to cause MED; wherein the entity has a direct or indirect effect on the level or activity of cAMP in the sexual genitalia of the male; and wherein said entity can be modulated to achieve a beneficial effect by use of an agent; and wherein said agent is an I:NPY.

35 25. A diagnostic composition or kit comprising means for detecting an entity in an isolated male sample; wherein the means can be used to determine whether the sample contains the entity and in such an amount to cause MED, or is in an amount so as to cause MED; wherein the entity has a direct or indirect effect on

the level or activity of cAMP in the sexual genitalia of the male and wherein said entity can be modulated to achieve a beneficial effect by use of an agent; and wherein said agent is an I:NPY.

- 5     26.     An animal model used to identify agents capable of treating MED, said model comprising an anaesthetised male animal including means to measure changes in genital blood flow of said animal following stimulation of the pelvic nerve thereof; and wherein said agent is an I:NPY.
- 10    27.     An assay method for identifying an agent that can directly or indirectly potentiate cAMP in order to treat MED, the assay method comprising: administering an agent to the animal model of claim 26; and measuring any potentiation of cAMP and/or increase in blood flow in the genital of said animal; and wherein said agent an I:NPY.
- 15     28.     An agent identified by the assay method according to claim 27; and wherein said agent is an I:NPY.
- 20     29.     A pharmaceutical composition for use (or when in use) in the treatment of MED; the pharmaceutical composition comprising an agent; wherein the agent is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient; and wherein said agent is an I:NPY.
- 25     30.     The use of an agent in the manufacture of a medicament (such as a pharmaceutical composition) for the treatment of MED; and wherein said agent is an I:NPY.
- 30     31.     A method of treating a male suffering from MED; the method comprising delivering to the male an agent; wherein the agent is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient; and wherein said agent is an I:NPY.
- 35     32.     A pharmaceutical composition for use (or when in use) in enhancing male genital blood flow; the pharmaceutical composition comprising an agent; wherein the agent is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient; and wherein said agent is an I:NPY.

33. The use of an agent in the manufacture of a medicament (such as a pharmaceutical composition) for enhancing male genital blood flow; and wherein said agent is an I:NPY.
- 5 34. A method of treating a male for MED or to prevent MED; the method comprising delivering to the male an agent; and wherein said agent is an I:NPY.
- 10 35. The invention according to any one of claims 29 to 34 wherein said agent potentiates cAMP.
36. The invention according to any one of the preceding claims wherein said cAMP is endogenous cAMP (as defined herein).
- 15 37. The invention according to any one of the preceding claims wherein said agent is an I:NPY Y1 or I:NPY Y2 or NPY Y5.
38. A pharmaceutical composition for use (or when in use) in the treatment of MED comprising an inhibitor of the NPY Y1 receptor optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient.
- 20 39. Use of a NPYi and a PDE5i for the treatment of MED.
40. Use according to claim 39 wherein the NPYi is an inhibitor of the NPY Y1 receptor and wherein the PDE5i is selected from:
- 25 5-[2-ethoxy-5-(4-methyl-1-piperazinylsulphonyl)phenyl]-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (sildenafil) also known as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulphonyl]-4-methylpiperazine;
- 30 5-(2-ethoxy-5-morpholinoacetylphenyl)-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;
- 35 3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-n-propoxyphenyl]-2-(pyridin-2-yl)methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-(2-methoxyethoxy)pyridin-3-yl]-2-(pyridin-2-yl)methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

(+)-3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-(2-methoxy-1(R)-methylethoxy)pyridin-3-yl]-2-methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one, also known as 3-ethyl-5-{5-[4-ethylpiperazin-1-ylsulphonyl]-2-[(1R)-2-methoxy-1-methylethyl]oxy}pyridin-3-yl}-2-methyl-2,6-dihydro-7H-pyrazolo[4,3-d] pyrimidin-7-one;

5-[2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-[2-methoxyethyl]-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one, also known as 1-{6-ethoxy-5-[3-ethyl-6,7-dihydro-2-(2-methoxyethyl)-7-oxo-2H-pyrazolo[4,3-d]pyrimidin-5-yl]-3-pyridylsulphonyl}-4-ethylpiperazine;

5-[2-*iso*-Butoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-(1-methylpiperidin-4-yl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

5-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-phenyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

20

5-(5-Acetyl-2-propoxy-3-pyridinyl)-3-ethyl-2-(1-isopropyl-3-azetidiny)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

5-(5-Acetyl-2-butoxy-3-pyridinyl)-3-ethyl-2-(1-ethyl-3-azetidiny)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

25

(6R,12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylenedioxyphenyl)pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione (IC-351);

2-[2-ethoxy-5-(4-ethyl-piperazin-1-yl-1-sulphonyl)-phenyl]-5-methyl-7-propyl-3H-imidazo[5,1-f][1,2,4]triazin-4-one (vardeafil) also known as 1-[[3-(3,4-dihydro-5-methyl-4-oxo-7-propylimidazo[5,1-f]-as-triazin-2-yl)-4-ethoxyphenyl]sulphonyl]-4-ethylpiperazine; and

the compound of example 11 of published international application WO93/07124 (EISA).

41. Use according to claim 40 wherein the PDE5i is sildenafil.
42. A pharmaceutical composition comprising an inhibitor of the NPY Y1 receptor and a PDE5i for the treatment of MED.
- 5 43. A kit comprising a first component and a second component for the treatment of MED wherein the first component comprises an inhibitor of the NPY Y1 receptor and wherein the second component comprises a PDE5i.



ABSTRACT

- 5 The present invention relates to the use of NPY inhibitors for the treatment of male sexual dysfunction, in particular MED.



## TREATMENT OF MALE SEXUAL DYSFUNCTION

### FIELD OF INVENTION

5 The present invention relates to compounds and pharmaceutical compositions for use in the treatment of male sexual dysfunction, in particular male erectile dysfunction (MED).

The present invention also relates to a method of treatment of MED.

10

The present invention also relates to assays to screen for the compounds of the present invention and which form part of the pharmaceutical compositions of the present invention and which are useful in the treatment of male sexual dysfunction, in particular MED.

15

For convenience, a list of abbreviations that are used in the following text is presented before the Claims section.

### SEXUAL DYSFUNCTION

20

Sexual dysfunction (SD) is a significant clinical problem which can affect both males and females. The causes of SD may be both organic as well as psychological. Organic aspects of SD are typically caused by underlying vascular diseases, such as those associated with hypertension or diabetes mellitus, by prescription medication and/or by psychiatric disease such as depression. Physiological factors include fear, performance anxiety and interpersonal conflict. SD impairs sexual performance, diminishes self-esteem and disrupts personal relationships thereby inducing personal distress. In the clinic, SD disorders have been divided into female sexual dysfunction (FSD) disorders and male sexual dysfunction (MSD) disorders (Melman *et al* 1999).

25

FSD is best defined as the difficulty or inability of a woman to find satisfaction in sexual expression. Male sexual dysfunction (MSD) is generally associated with erectile dysfunction, also known as male erectile dysfunction (MED) (Benet *et al* 1994).

30

35

## MALE ERECTILE DYSFUNCTION (MED)

It is known that some individuals can suffer from male erectile dysfunction (MED).

5 Male erectile dysfunction (MED) is defined as:

“the inability to achieve and/or maintain a penile erection for satisfactory sexual performance” (NIH Consensus Development Panel on Impotence, 1993)”

10 It has been estimated that the prevalence of erectile dysfunction (ED) of all degrees (minimal, moderate and complete impotence) is 52% in men 40 to 70 years old, with higher rates in those older than 70 (Melman *et al* 1999). The condition has a significant negative impact on the quality of life of the patient and their partner, often  
15 resulting in increased anxiety and tension which leads to depression and low self esteem. Whereas two decades ago, MED was primarily considered to be a psychological disorder (Benet *et al* 1994), it is now known that for the majority of patients there is an underlying organic cause. As a result, much progress has been made in identifying the mechanism of normal penile erection and the pathophysiology of MED.

20

Penile erection is a haemodynamic event which is dependent upon the balance of contraction and relaxation of the corpus cavernosal smooth muscle and vasculature of the penis (Lerner *et al* 1993). Corpus cavernosal smooth muscle is also referred to herein as corporal smooth muscle or in the plural sense corpus cavernosa.

25 Relaxation of the corpus cavernosal smooth muscle leads to an increased blood flow into the trabecular spaces of the corpus cavernosa, causing them to expand against the surrounding tunica and compress the draining veins. This produces a vast elevation in blood pressure which results in an erection (Naylor, 1998).

30 The changes that occur during the erectile process are complex and require a high degree of co-ordinated control involving the peripheral and central nervous systems, and the endocrine system (Naylor, 1998). Corporal smooth muscle contraction is modulated by sympathetic noradrenergic innervation via activation of postsynaptic  $\alpha_1$  adrenoceptors. MED may be associated with an increase in the endogenous smooth  
35 muscle tone of the corpus cavernosum. However, the process of corporal smooth muscle relaxation is mediated primarily by non-adrenergic, non-cholinergic (NANC) neurotransmission. There are a number of other NANC neurotransmitters found in

the penis, other than NO, such as calcitonin gene related peptide (CGRP) and vasoactive intestinal peptide (VIP). The main relaxing factor responsible for mediating this relaxation is nitric oxide (NO), which is synthesised from L-arginine by nitric oxide synthase (NOS) (Taub *et al* 1993; Chuang *et al* 1998). It is thought that reducing corporal smooth muscle tone may aid NO to induce relaxation of the corpus cavernosum. During sexual arousal in the male, NO is released from neurones and the endothelium and binds to and activates soluble guanylate cyclase (sGC) located in the smooth muscle cells and endothelium, leading to an elevation in intracellular cyclic guanosine 3',5'-monophosphate (cGMP) levels. This rise in cGMP leads to a relaxation of the corpus cavernosum due to a reduction in the intracellular calcium concentration ( $[Ca^{2+}]_i$ ), via unknown mechanisms thought to involve protein kinase G activation (possibly due to activation of  $Ca^{2+}$  pumps and  $Ca^{2+}$ -activated  $K^+$  channels; Chuang *et al.*, 1998).

MED mainly arises from an inability of NO released during sexual arousal to effectively relax corpus cavernosal smooth muscle. It has therefore been proposed that MED may be treatable by potentiating or facilitating nitrgenic signalling thereby leading to an elevation in intracellular cGMP levels. Nitrgenic signalling as defined herein means the cellular mechanisms that are activated by the NO released or generated during sexual arousal / stimulation and in particular relates to the activation of the guanlyl cyclase / cGMP pathway. In this respect, sildenafil citrate (also known as Viagra<sup>TM</sup>) has recently been developed by Pfizer as the first oral drug treatment for MED. Sildenafil acts by inhibiting cGMP breakdown in the corpus cavernosa by selectively inhibiting phosphodiesterase 5 (PDE5), thereby limiting the hydrolysis of cGMP to 5'GMP (Boolel *et al.*, 1996; Jeremy *et al.*, 1997) and thereby increasing the intracellular concentrations of cGMP and facilitating corpus cavernosal smooth muscle relaxation.

Currently, all other available MED therapies on the market, such as treatment with prostaglandin based compounds i.e. alprostadil which can be administered intra-urethrally (available from Vivus Inc., as Muse<sup>TM</sup>) or via small needle injection (available from Pharamcia & Upjohn, as Caverject<sup>TM</sup>), are either inconvenient and/or invasive. Other treatments include vacuum constriction devices, vasoactive drug injection or penile prostheses implantation (Montague *et al.*, 1996). Although injectable vasoactive drugs show high efficacy, side effects such as penile pain, fibrosis and priapism are common, and injection therapy is not as convenient as oral

therapy therefore sildenafil currently represents the most preferred therapy on the market.

The present invention relates to compounds and pharmaceutical compositions useful for the treatment of MED which act via a complementary pathway to the inhibition of cGMP breakdown by PDE enzyme hydrolysis. A complementary pathway(s) as defined herein means a pathway which is implicated in the endogenous erectile process and is stimulated by sexual arousal. Further, a complementary pathway as defined herein is substantially, and preferably wholly, independent of the release of NO from nitrergic nerves by sexual stimulation. Moreover the complementary pathway as defined herein exists in complement with the nitrergic/NO pathway which is also stimulated by sexual arousal. In other words the complementary pathway means a pathway working via a separate mechanism to the PDE5 enzyme-mediated hydrolysis of cGMP (as discussed hereinbefore in relation to sildenafil) wherein said complementary pathway essentially acts via a non-soluble guanylate cyclase (nsGC) activated pathway. Acting essentially via a non-soluble nsGC activated pathway means that the majority of action is via non-soluble nsGC whilst a minority may act via soluble guanylate cyclase (sGC). The terms majority and minority are relative terms wherein a majority is preferably greater than about 80%, more preferably greater than about 90% and especially greater than about 95%.

It has previously been proposed that mechanisms other than inhibition of cGMP breakdown, such as, mechanisms involving neuropeptides (e.g. endothelin-1, vasoactive intestinal peptide (VIP); (Christ *et al.*, 1995), gap junctions (Christ *et al.*, 1991) and ion channels (Christ *et al.*, 1993; Chiou *et al.*, 1998) may also modulate corporal smooth muscle tone.

VIP is localised to the neurones innervating the corpus cavernosa and penile vasculature (Argiolas *et al.*, 1995). However the role of VIP in the erectile mechanism is less clear, compared to that for NO. Whilst it has been proposed that VIP may have a role in the erectile process clinical data to date has been negative. Exogenously applied VIP potently relaxes isolated human corpus cavernosum muscle strips (Willis *et al.*, 1983; Steers *et al.*, 1984, Hedlund *et al.*, 1985, Adaikan *et al.*, 1986). Despite these results in isolated tissues, repeated clinical studies have shown that intracavernosal injection of VIP does not produce erections in normal or impotent men (Wagner *et al.*, 1987; Kiely *et al.*, 1989; Roy *et al.*, 1990). Furthermore, there is evidence against the involvement of VIP as the key NANC

transmitter of erection (reviewed in Naylor, 1998). Whilst the art teaches that administration of VIP alone, with, or without sexual stimulation, to a male suffering from MED does not produce erection sufficient for intercourse, it has been reported that combinations of VIP with phentolamine or papaverine can induce erections sufficient for intercourse, in the absence of sexual stimulation, when administered intracavernosally, (Kiely *et al.*, 1989). Phentolamine is an  $\alpha$ -adrenergic antagonist and a known treatment for MED (available as Vasomax<sup>TM</sup> from Zonagen Inc.). It has been proposed that the erections observed for VIP/phentolamine or papaverine combinations were due to increases in venous outflow resistance attributable to  $\alpha$ -adrenergic antagonists i.e. phentolamine or papaverine rather than a VIP-induced effect.

The present invention provides compounds and pharmaceutical compositions useful for the treatment of MED which act via a complementary pathway to the inhibition of cGMP breakdown by PDE enzyme hydrolysis.

Surprisingly the applicants have also found that inhibition of NEP EC3.4.24.11 with a selective neural endopeptidase inhibitor, hereinafter referred to as an NEPi, significantly enhances the nerve-stimulated erectile process.

According to the present invention there is provided the use of a selective inhibitor of the neural endopeptidase EC3.4.24.11, hereinafter referred to as a NEPi, for the treatment of MED.

There is further provided the use of a NEPi in the manufacture of a medicament for the treatment of MED.

There is no documented evidence for the expression or a functional role of NEP EC3.4.24.11 in the penis or corpus cavernosum or in the erectile mechanism/process.

There is no documented evidence for a functional or biochemical effect for selective NEP inhibitors on the penis or corpus cavernosum or alternatively in the erectile mechanism/process.

### Summary Aspects of The Present Invention

A seminal finding of the present invention is the ability to treat an male suffering from sexual dysfunction, in particular MED, with use of an NEPi.

The present invention provides compounds and pharmaceutical compositions useful for the treatment of MED which act via a complementary pathway to the inhibition of the cGMP breakdown by PDE enzyme hydrolysis.

In particular the present invention provides NEPi compounds for use in the treatment of MED.

The present invention is advantageous as it provides a means for restoring a normal sexual arousal response - namely increased penile blood flow leading to erection of the penis. Hence, the present invention provides a means to restore, or potentiate, the normal sexual arousal response.

Without wishing to be bound to any particular theory it is believed that use of the compounds of the present invention act via inhibiting the mechanism that terminates the biological activity of a number of bioactive peptides and in particular vasoactive peptides, more particularly neuropeptides, that are released during sexual arousal in order to treat MED wherein said mechanism is a complementary pathway to the NO pathway as defined hereinbefore. Vasoactive peptides include both vasorelaxant and vasoconstrictor peptides, preferred.

It is further proposed that the use of the compounds according to the present invention acts via enhancing a non-NO dependant NANC pathway to treat MED.

It is proposed herein that the use of NEPi compounds for the treatment of MED according to the present invention acts upon a complementary pathway, as defined hereinbefore, or acts to potentiate or facilitate upon said complementary pathway. By this reasoning it is further proposed that the use of NEP inhibitor compounds for the treatment of MED may be advantageous via their potential to overcome the deficiency/reduction in nitrgenic signalling by acting upon said complementary pathway.



Surprisingly the applicants have also found that inhibition of NEP EC3.4.24.11 with a selective neural endopeptidase inhibitor, hereinafter referred to as an NEPi, significantly potentiates PDE5 inhibitor-mediated enhancement of the erectile process.

5

It is therefore an object of the present invention to provide NEPi compounds and pharmaceutical compositions comprising NEPi compounds that inhibit NEP EC3.4.24.11 for use in the treatment of MED. For examples and discussion thereof see Test Results Section, Examples 1, 2 and 3.

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Thus according to a further embodiment the present invention provides the use of one or more NEPi's and one or more PDE5i's for the treatment of MED.

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Preferably said combined treatment comprises a combination of one or more NEPi's with one or more PDE5i's. More preferably such combination provides for the concomitant administration of one or more NEPi's with one or more PDE5i's for the treatment of MED.

20

Highly preferred herein is the use of a pharmaceutical composition comprising one or more NEPi's with one or more PDE5i's for the treatment of MED.

25

Especially preferred for use in the pharmaceutical compositions for the treatment of MED according to the present invention is the combination of a potent and selective NEPi with a potent and selective PDE5i.

30

In a preferred embodiment herein said combined administration of NEPi and PDE5i is concomitant. Concomitant administration as defined herein encompasses simultaneous (separate) administration, simultaneous combined administration, separate administration, combined administration, sequential administration and co-formulated combined administration of a cGMP PDE5i and a NEPi.

35

As detailed hereinbefore the present invention further proposes that, concomitant administration of a PDE5i and NEPi can effect an increase in the efficacy vs that obtainable by PDE5-alone associated MED therapy. For example and discussion thereof see Test Results Section, Examples 4 and 5.

Thus, it is a further object of the present invention to provide pharmaceutical compositions comprising an NEPi and a PDE5i for use in the treatment of MED.

According to a further aspect of the present invention it is proposed herein that, concomitant application of an NEPi and a PDE5i can provide faster onset of action that that achievable via the PDE5i alone. In other words the present invention additionally provides the use of a fast-acting composition for the treatment of MED. A fast acting MED composition as defined herein, and as exemplified hereinafter, means that following i.v. administration of the composition (NEPi and PDE5i) the time to maximal effect on intracavernosal pressure is reduced versus the equivalent time obtained for the same dose of the PDE5i alone. For example and discussion thereof see Test Results Section, Examples 5

Thus, it is a yet further object of the present invention to provide fast acting pharmaceutical compositions comprising an NEPi and a PDE5i for use in the treatment of MED.

It is further proposed herein that use of a NEPi/PDE5i combination may enhance the efficacy of the PDE5i thereby enabling a reduction in the dose of PDE5 inhibitor required for a specific efficacy. A formulation comprising a NEPi and a reduced amount of a PDE5i as defined herein means that a reduced amount of a given PDE5i is required to effect a particular response when combined with an effective amount of a NEPi according to the present invention than the required amount of PDE5i alone. Such reduced dose compositions for the treatment of MED may be desirable for particular patient groups such as for example men with mild MED.

Thus, it is a still further object of the present invention to provide a pharmaceutical composition comprising an NEPi and a reduced dose of PDE5i for use in the treatment of MED.

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#### NEP EC3.4.24.11

NEP EC3.4.24.11, also known as enkephalinase or neprilysin, is a zinc-dependent neutral endopeptidase. This enzyme is involved in the breakdown of several bioactive oligopeptides, cleaving peptide bonds on the amino side of hydrophobic amino acid residues (Reviewed in Turner et al., 1997). The key neuronally released bioactive agents or neuropeptides metabolised by NEP include natriuretic peptides

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such as atrial natriuretic peptides (ANP) as well as brain natriuretic peptide and C-type natriuretic peptide, bombesin, bradykinin, calcitonin gene-related peptide, endothelins, enkephalins, neurotensin, substance P and vasoactive intestinal peptide. Some of these peptides have potent vasodilatory and neurohormone functions, diuretic and natriuretic activity or mediate behaviour effects.

Without being limited to any particular theory it is proposed herein that by inhibiting NEP EC3.4.24.11 other neuronally released vasoactive agents that are released during sexual arousal are enhanced, most likely VIP. It is further proposed herein that the complementary effects of NEP substrates or substrates of NEP metabolism, most likely VIP or other bioactive peptides, with the NO/cGMP pathway that is responsible for the observed effects described for the use of the compounds and pharmaceutical combinations according to the present invention for the treatment of MED.

The NEPi, and where present PDE5i compounds, useful for the treatment of MED according to the present invention, may also be used in combination with one or more additional pharmaceutically active agents. The additional pharmaceutically active agent(s) as defined hereinbefore, if present, may be referred to as an "additional agent". One or more of such additional agents may be one or more of: PDEi, another NEPi, or an NPYi. Combinations of agents are discussed in more detail below.

General references herein to agents may be applicable to additional agents as well as to NEPi or PDE5i compounds.

In accordance with the use of NEPi compounds for the treatment of MED according to the as discussed hereinbefore, the NEPi acts on a target, preferably specifically on that target. For example where a combination of a NEPi and a PDE5i are present the targets are the NEP and PDE5 enzymes. This target is sometimes referred to as the "target of the present invention". However, the additional agents of the present invention may act on one or more other targets. These other targets may be referred to as an "additional target". Likewise, if an additional agent is used, then that additional agent can target the same target of the present invention and/or an additional target (which need not be the same additional target that is acted on by the agent of the present invention). Targets are described herein. It is to be understood

that general references herein to targets may be applicable to the additional targets as well as to the target of the present invention.

5     DETAILED ASPECTS OF THE PRESENT INVENTION

In one aspect, the present invention relates to NEPi compounds and pharmaceutical compositions including NEPi compounds and pharmaceutical combinations comprising NEPi and PDE5i for use (or when in use) in the treatment of male sexual  
10     dysfunction, in particular MED. In said pharmaceutical compositions the NEPi (and PDE5i, if present, and/or additional agent) is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient. Here, the composition (like any of the other compositions mentioned herein) may be packaged for subsequent use in the treatment of male sexual dysfunction, in particular MED.

15

In another aspect, the present invention relates to the use of an agent in the manufacture of a medicament (such as a pharmaceutical composition) for the treatment of male sexual dysfunction, in particular MED.

20     In a further aspect, the present invention relates to a method of treating a male suffering from male sexual dysfunction, in particular MED; the method comprising delivering to the male an NEPi that is capable of enhancing the endogenous erectile process in the corpus cavernosum; wherein the NEPi is present in an amount to enhance the endogenous erectile process as defined hereinbefore; wherein the NEPi  
25     is optionally admixed with a pharmaceutically acceptable carrier, diluent or excipient; and wherein said NEPi is as herein defined.

In a further aspect, the present invention relates to an assay method for identifying an agent (hereinafter referred to as a NEPi) that can be used to treat male sexual  
30     dysfunction, in particular MED, the assay method comprising: determining whether a test agent can directly enhance the endogenous erectile process; wherein said enhancement is defined as a potentiation of intracavernosal pressure (ICP) (and/or cavernosal blood flow) in the presence of a test agent as defined herein; such potentiation by a test agent is indicative that the test agent may be useful in the  
35     treatment of male sexual dysfunction, in particular MED and wherein said test agent is a NEPi.

By way of example, the present invention relates to an assay method for identifying an agent that can directly enhance the endogenous erectile process in order to treat male sexual dysfunction, in particular MED, the assay method comprising: contacting a test agent with a moiety capable of inhibiting the metabolic breakdown of a peptide (preferably a fluorescent labelled peptide); and measuring the activity and/or levels of peptide remaining after a fixed time (for example via fluorometric analysis); wherein the change in the level of fluorescence is indicative of the potency ( $IC_{50}$ ) of the test agent and is indicative that the test agent may be useful in the treatment of male sexual dysfunction, in particular MED; and wherein said agent is an NEPi.

In a further aspect, the present invention relates to a process comprising the steps of: (a) performing the assay according to the present invention; (b) identifying one or more agents that can directly enhance the endogenous erectile process; and (c) preparing a quantity of those one or more identified agents; and wherein said agent is an NEPi.

With this aspect, the agent identified in step (b) may be modified so as to, for example, maximise activity and then step (a) may be repeated. These steps may be repeated until the desired activity or pharmacokinetic profile has been achieved.

Thus, in a further aspect, the present invention relates to a process comprising the steps of: (a1) performing the assay according to the present invention; (b1) identifying one or more agents that can directly enhance the endogenous erectile process ; (b2) modifying one or more of said identified agents; (a2) optionally repeating step (a1); and (c) preparing a quantity of those one or more identified agents (i.e. those that have been modified); and wherein said agent is an NEPi.

In a further aspect, the present invention relates to a method of treating male sexual dysfunction, in particular MED, by potentiating the nerve stimulated endogenous erectile process *in vivo* (rabbit and / or dog) by measuring the ICP or cavernosal blood flow with an agent; wherein the agent is capable of directly inhibiting the metabolic breakdown of a fluorescent peptide (as detailed hereinbefore) in an *in vitro* assay method; wherein the *in vitro* assay method is the assay method according to the present invention; and wherein said agent is an NEPi.

In a further aspect, the present invention relates to the use of an agent in the preparation of a pharmaceutical composition for the treatment of male sexual dysfunction, in particular MED, wherein the agent is capable of directly inhibiting the

metabolic breakdown of a fluorescent peptide when assayed *in vitro* by the assay method according to the present invention; and wherein said agent is an NEPi.

5 In a further aspect, the present invention relates to an animal model used to identify agents capable of treating male sexual dysfunction (in particular MED), said model comprising an anaesthetised male animal including means to measure changes in intracavernosal pressure and/or cavernosal blood flow of said animal following stimulation of the pelvic nerve thereof; and wherein said agent is an NEPi.

10 In a further aspect, the present invention relates to an assay method for identifying an agent that can directly enhance the endogenous erectile process in order to treat MED, the assay method comprising: administering an agent to the animal model of the present invention; and measuring the change in the endogenous erectile process; wherein said change is defined as a potentiation of intracavernosal pressure (ICP)  
15 (and/or cavernosal blood flow) in the animal model in the presence of a test agent as defined; and wherein said agent is an NEPi.

In a further aspect, the present invention relates to a diagnostic method, the method comprising isolating a sample from a male; determining whether the sample contains an  
20 entity present in such an amount as to cause male sexual dysfunction, preferably MED; wherein the entity has a direct effect on the endogenous erectile process in the corpus cavernosum of the male; and wherein said entity can be modulated to achieve a beneficial effect by use of an agent; and wherein said agent is an NEPi.

25 In a further aspect, the present invention relates to a diagnostic composition or kit comprising means for detecting an entity in an isolated male sample; wherein the means can be used to determine whether the sample contains the entity and in such an amount to cause male sexual dysfunction, preferably MED, or is in an amount so as to cause sexual dysfunction, preferably MED; wherein the entity has a direct effect on the  
30 endogenous erectile process and wherein said entity can be modulated to achieve a beneficial effect by use of an agent; and wherein said agent is an NEPi.

For ease of reference, these and further aspects of the present invention are now discussed under appropriate section headings. However, the teachings under each  
35 section are not necessarily limited to each particular section.

#### PREFERABLE ASPECTS

Preferably, the agents for use in the treatment of MED according to the present invention are NEP EC3.4.24.11 inhibitors.

- 5 In one embodiment, preferably the agent for the use according to the present invention may be used via oral administration.

In another embodiment, the agent for the use according to the present invention may be used via topical or intra-urethral, preferably topical administration.

10

For some applications, preferably the agent for the use according to the present invention is a selective NEPi.

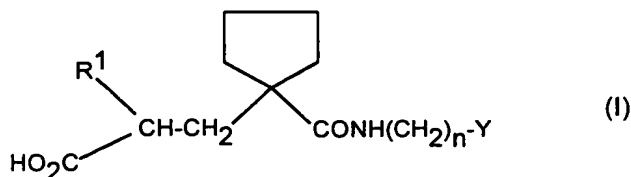
- 15 For some applications, preferably the agent for use herein is an NEPi wherein said NEP is EC 3.4.24.11.

For some applications, preferably the agent for use herein is a selective NEPi wherein said NEP is EC 3.4.24.11.

- 20 Preferably the agent for use in the treatment of MED according to the present invention is an inhibitor – i.e. it is capable of exhibiting an inhibitory function.

- 25 Preferably the agent for use in the treatment of MED according to the present invention is capable of directly enhancing the endogenous erectile process as detailed hereinbefore.

Preferred for use as NEPi's in the treatment of MED according to the present invention are compounds of general formula I:



30

wherein

R<sup>1</sup> is C<sub>1-6</sub>alkyl which may be substituted by one or more substituents, which may be the same or different, selected from the list: halo, hydroxy, C<sub>1-6</sub> alkoxy, C<sub>2-6</sub>

hydroxyalkoxy, C<sub>1-6</sub> alkoxy(C<sub>1-6</sub>alkoxy), C<sub>3-7</sub>cycloalkyl, C<sub>3-7</sub>cycloalkenyl, aryl, aryloxy, (C<sub>1-4</sub>alkoxy)aryloxy, heterocyclyl, heterocyclyloxy, -NR<sup>2</sup>R<sup>3</sup>, -NR<sup>4</sup>COR<sup>5</sup>, -NR<sup>4</sup>SO<sub>2</sub>R<sup>5</sup>, -CONR<sup>2</sup>R<sup>3</sup>, -S(O)<sub>p</sub>R<sup>6</sup>, -COR<sup>7</sup> and -CO<sub>2</sub>(C<sub>1-4</sub>alkyl); or R<sup>1</sup> is C<sub>3-7</sub>cycloalkyl, aryl or heterocyclyl, each of which may be substituted by one or more substituents from said list, which substituents may be the same or different, which list further includes C<sub>1-6</sub>alkyl; or R<sup>1</sup> is C<sub>1-6</sub> alkoxy, -NR<sup>2</sup>R<sup>3</sup> or -NR<sup>4</sup>SO<sub>2</sub>R<sup>5</sup>;

wherein

R<sup>2</sup> and R<sup>3</sup> are each independently H, C<sub>1-4</sub>alkyl, C<sub>3-7</sub>cycloalkyl (optionally substituted by hydroxy or C<sub>1-4</sub>alkoxy), aryl, (C<sub>1-4</sub>alkyl)aryl, C<sub>1-6</sub>alkoxyaryl or heterocyclyl; or R<sup>2</sup> and R<sup>3</sup> together with the nitrogen to which they are attached form a pyrrolidinyl, piperidino, morpholino, piperazinyl or N-(C<sub>1-4</sub> alkyl)piperazinyl group;

R<sup>4</sup> is H or C<sub>1-4</sub>alkyl;

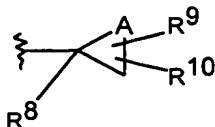
R<sup>5</sup> is C<sub>1-4</sub>alkyl, CF<sub>3</sub>, aryl, (C<sub>1-4</sub> alkyl)aryl, (C<sub>1-4</sub>alkoxy)aryl, heterocyclyl, C<sub>1-4</sub>alkoxy or -NR<sup>2</sup>R<sup>3</sup> wherein R<sup>2</sup> and R<sup>3</sup> are as previously defined;

R<sup>6</sup> is C<sub>1-4</sub>alkyl, aryl, heterocyclyl or NR<sup>2</sup>R<sup>3</sup> wherein R<sup>2</sup> and R<sup>3</sup> are as previously defined; and

R<sup>7</sup> is C<sub>1-4</sub>alkyl, C<sub>3-7</sub>cycloalkyl, aryl or heterocyclyl; n is 0, 1 or 2; p is 0, 1, 2 or 3;

the -(CH<sub>2</sub>)<sub>n</sub>- linkage is optionally substituted by C<sub>1-4</sub>alkyl, C<sub>1-4</sub>alkyl substituted with one or more fluoro groups or phenyl, C<sub>1-4</sub>alkoxy, hydroxy, hydroxy(C<sub>1-3</sub>alkyl), C<sub>3-7</sub>cycloalkyl, aryl or heterocyclyl;

Y is the group



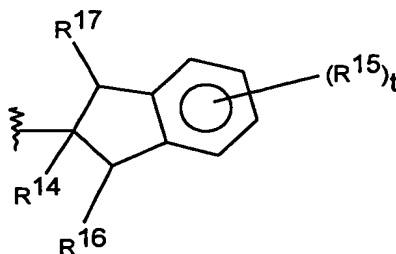
wherein A is -(CH<sub>2</sub>)<sub>q</sub>- where q is 1, 2, 3 or 4 to complete a 3 to 7 membered carbocyclic ring which may be saturated or unsaturated; R<sup>8</sup> is H, C<sub>1-6</sub>alkyl, -CH<sub>2</sub>OH, phenyl, phenyl(C<sub>1-4</sub>alkyl) or CONR<sup>11</sup>R<sup>12</sup>; R<sup>9</sup> and R<sup>10</sup> are each



independently H,  $-\text{CH}_2\text{OH}$ ,  $-\text{C}(\text{O})\text{NR}^{11}\text{R}^{12}$ ,  $\text{C}_{1-6}\text{alkyl}$ , phenyl optionally substituted by  $\text{C}_{1-4}\text{alkyl}$ , or phenyl( $\text{C}_{1-4}\text{alkyl}$ ) wherein the phenyl group is optionally substituted by  $\text{C}_{1-4}\text{alkyl}$ , or  $\text{R}^9$  and  $\text{R}^{10}$  together form a dioxolane;  $\text{R}^{11}$  and  $\text{R}^{12}$  which may be the same or different are H,  $\text{C}_{1-4}\text{alkyl}$ ,  $\text{R}^{13}$  or  $\text{S}(\text{O})_r\text{R}^{13}$ , where  $r$  is 0, 1 or 2 and  $\text{R}^{13}$  is phenyl optionally substituted by  $\text{C}_{1-4}\text{alkyl}$  or phenyl $\text{C}_{1-4}\text{alkyl}$  wherein the phenyl is optionally substituted by  $\text{C}_{1-4}\text{alkyl}$ ; or

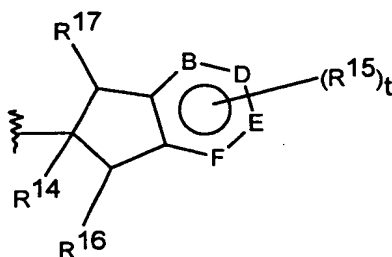
Y is the group,  $-\text{C}(\text{O})\text{NR}^{11}\text{R}^{12}$  wherein  $\text{R}^{11}$  and  $\text{R}^{12}$  are as previously defined except that  $\text{R}^{11}$  and  $\text{R}^{12}$  are not both H; or

10 Y is the group,



wherein  $\text{R}^{14}$  is H,  $\text{CH}_2\text{OH}$ , or  $\text{C}(\text{O})\text{NR}^{11}\text{R}^{12}$  wherein  $\text{R}^{11}$  and  $\text{R}^{12}$  are as previously defined; when present  $\text{R}^{15}$ , which may be the same or different to any other  $\text{R}^{15}$ , is OH,  $\text{C}_{1-4}\text{alkyl}$ ,  $\text{C}_{1-4}\text{alkoxy}$ , halo or  $\text{CF}_3$ ;  $t$  is 0, 1, 2, 3 or 4; and  $\text{R}^{16}$  and  $\text{R}^{17}$  are independently H or  $\text{C}_{1-4}\text{alkyl}$ ; or

Y is the group



wherein one or two of B, D, E or F is a nitrogen, the others being carbon; and  $\text{R}^{14}$  to  $\text{R}^{17}$  and  $t$  are as previously defined; or

20 Y is an optionally substituted 5-7 membered heterocyclic ring, which may be saturated, unsaturated or aromatic and contains a nitrogen, oxygen or sulphur and optionally one, two or three further nitrogen atoms in the ring and which may be optionally benzofused and optionally substituted by:

C<sub>1-6</sub> alkoxy; hydroxy; oxo; amino; mono or di-(C<sub>1-4</sub>alkyl)amino;  
C<sub>1-4</sub>alkanoylamino; or

C<sub>1-6</sub>alkyl which may be substituted by one or more substituents, which may  
be the same or different, selected from the list: C<sub>1-6</sub>alkoxy, C<sub>1-6</sub>  
haloalkoxy, C<sub>1-6</sub>alkylthio, halogen, C<sub>3-7</sub>cycloalkyl, heterocyclyl or  
phenyl; or

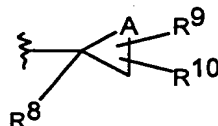
C<sub>3-7</sub>cycloalkyl, aryl or heterocyclyl, each of which may be substituted by one  
or more substituents, which may be the same or different, selected  
from the list: C<sub>1-6</sub>alkyl, C<sub>1-6</sub>alkoxy, C<sub>1-6</sub>haloalkoxy, C<sub>1-6</sub>alkylthio,  
halogen, C<sub>3-7</sub>cycloalkyl, heterocyclyl or phenyl;

wherein when there is an oxo substitution on the heterocyclic ring, the ring  
only contains one or two nitrogen atoms and the oxo substitution is adjacent a  
nitrogen atom in the ring; or

Y is -NR<sup>18</sup>S(O)<sub>u</sub>R<sup>19</sup>, wherein R<sup>18</sup> is H or C<sub>1-4</sub>alkyl; R<sup>19</sup> is aryl, arylC<sub>1-4</sub>alkyl or  
heterocyclyl (preferably pyridyl); and  
u is 0, 1, 2 or 3.

Preferably R<sup>1</sup> is C<sub>1-6</sub>alkyl, C<sub>1-6</sub>alkoxy, C<sub>1-6</sub>alkoxy(C<sub>1-3</sub>)alkyl,  
C<sub>1-6</sub>alkoxyC<sub>1-6</sub>alkoxyC<sub>1-3</sub>alkyl or C<sub>1-6</sub>alkyl substituted with aryl. Particularly  
preferred R<sup>1</sup> substituents are C<sub>1-6</sub>alkyl, C<sub>1-6</sub>alkoxy, C<sub>1-6</sub>alkoxy(C<sub>1-3</sub>)alkyl  
(especially methoxyethyl) or C<sub>1-6</sub>alkoxyC<sub>1-6</sub>alkoxyC<sub>1-3</sub>alkyl (especially  
methoxyethoxymethyl). It is especially preferred that R<sup>1</sup> is C<sub>1-4</sub>alkyl (preferably  
propyl).

When Y is the group



and the carbocyclic ring is fully saturated, then preferably one of R<sup>9</sup> or R<sup>10</sup> is  
-CH<sub>2</sub>OH, -C(O)NR<sup>11</sup>R<sup>12</sup>, C<sub>1-6</sub>alkyl, phenyl optionally substituted by C<sub>1-4</sub>alkyl or  
phenyl(C<sub>1-4</sub>alkyl) wherein the phenyl group is optionally substituted by C<sub>1-4</sub>alkyl.

More, preferably the carbocyclic ring is 5, 6 or 7 membered wherein one of R<sup>9</sup> or  
R<sup>10</sup>, -C(O)NR<sup>11</sup>R<sup>12</sup>, with the other being C<sub>1-6</sub>alkyl, phenyl optionally substituted by

C<sub>1-4</sub>alkyl or phenyl(C<sub>1-4</sub>alkyl) wherein the phenyl group is optionally substituted by C<sub>1-4</sub>alkyl. More preferably, R<sup>9</sup> and R<sup>10</sup> are attached to adjacent carbon atoms in the ring. More preferably, R<sup>8</sup> is CH<sub>2</sub>OH.

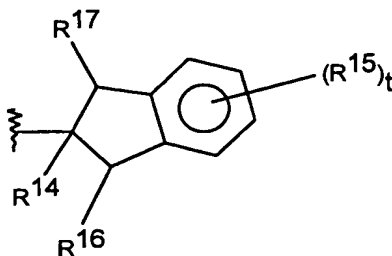
- 5 When Y is the group -NR<sup>18</sup>S(O)<sub>u</sub>R<sup>19</sup>, preferably R<sup>18</sup> is H. More preferably, R<sup>19</sup> is benzyl or phenyl. More preferably u is 2.

- Preferably Y is the optionally substituted 5-7 membered heterocyclic ring. More preferably the ring is an optionally substituted aromatic ring, particularly pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, pyrazolyl, triazolyl, tetrazolyl, oxadiazolyl, thiazolyl, thiaiazolyl, oxazolyl, isoxazolyl, indolyl, isoindolyl, quinolyl, isoquinolyl, pyridonyl, quinoxaliny or quinazolinyl [especially oxadiazole (preferably 1,2,5- or 1,3,4-oxadiazole), pyridone (preferably 2-pyridone) or thiadiazole (preferably 1,3,4-thiadiazole) each of which may be substituted as defined in the first aspect.
- 10 Preferably the heterocyclic ring is substituted by one or more C<sub>1-6</sub>alkyl, phenyl or phenylC<sub>1-4</sub>alkyl, more preferably by C<sub>1-4</sub>alkyl or benzyl. Preferably Y is an *N*-substituted pyridone, preferably by benzyl or C<sub>1-4</sub>alkyl.
- 15

Preferably Y is a lactam linked at the nitrogen.

20

Preferably Y is



wherein R<sup>14</sup> is preferably CH<sub>2</sub>OH or C(O)NR<sup>11</sup>R<sup>12</sup>, especially C(O)NR<sup>11</sup>R<sup>12</sup>.

- 25 Preferably R<sup>16</sup> and R<sup>17</sup> are hydrogen. Preferably t is 0.

The chiral carbon attached to R<sup>1</sup> is preferably the R-enantiomer.

Particularly preferred NEPi compounds for use in the treatment of MED according to the present invention (referred to hereinafter as the list of 10 preferred NEPi compounds) are:

- 5 2-[(1-[(1-benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl)cyclopentyl)methyl]-4-methoxybutanoic acid (NEPi Example 35),
- 2-[(1-[(3-(2-oxo-1-pyrrolidinyl)propyl)amino]carbonyl)cyclopentyl]-methyl]-4-phenylbutanoic acid (NEPi Example 40),
- (+)-2-[(1-[(2-(hydroxymethyl)-2,3-dihydro-1*H*-inden-2-yl)amino]carbonyl)cyclopentyl]-methyl]-4-phenylbutanoic acid (NEPi Example 44),
- 10 2-[(1-[(5-methyl-1,3,4-thiadiazol-2-yl)amino]carbonyl)cyclopentyl)methyl]-4-phenylbutanoic acid (NEPi Example 43),
- cis-3-(2-methoxyethoxy)-2-[(1-[(4-[(phenylsulfonyl)amino]carbonyl)cyclohexyl]-amino]carbonyl)cyclopentyl)methyl]propanoic acid (NEPi Example 38),
- (+)-2-[(1-[(2-(hydroxymethyl)-2,3-dihydro-1*H*-inden-2-yl)amino]carbonyl)cyclopentyl]-methyl]pentanoic acid (NEPi Example 31),
- 15 (+)-2-[(1-[(5-ethyl-1,3,4-thiadiazol-2-yl)amino]carbonyl)cyclopentyl)methyl]pentanoic acid (NEPi Example 30),
- 2-[(1-[(3-benzylanilino)carbonyl)cyclopentyl)methyl]pentanoic acid (Example 21),
- 2-[(1-[(1-benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl)cyclopentyl)methyl]-pentanoic acid (NEPi Example 22), and
- 20 2-[(1-[(1*R*,3*S*,4*R*)-4-(aminocarbonyl)-3-butylcyclohexyl)amino]carbonyl)-cyclopentyl)methyl]pentanoic acid (NEPi Example 9).

25 In the above definition, unless otherwise indicated, alkyl groups having three or more carbon atoms may be straight or branched-chain. The term aryl as used herein means an aromatic hydrocarbon group such as phenyl or naphthyl which may optionally be substituted with, for example, one or more of OH, CN, CF<sub>3</sub>, C<sub>1</sub>-C<sub>4</sub> alkyl, C<sub>1</sub>-C<sub>4</sub> alkoxy, halo, carbamoyl, aminosulphonyl, amino, mono or di(C<sub>1</sub>-C<sub>4</sub> alkyl)amino or (C<sub>1</sub>-C<sub>4</sub> alkanoyl)amino groups. Halo means fluoro, chloro, bromo or  
30 iodo.

In the above definition, unless otherwise indicated the term heterocyclyl means a 5 or 6 membered nitrogen, oxygen or sulphur containing heterocyclic group which, unless  
35 optionally include a further oxygen or one to three nitrogen atoms in the ring and which may optionally be benzofused or substituted with for example, one or more

halo, C<sub>1</sub>-C<sub>4</sub> alkyl, hydroxy, carbamoyl, benzyl, oxo, amino or mono or di-(C<sub>1</sub>-C<sub>4</sub> alkyl)amino or (C<sub>1</sub>-C<sub>4</sub> alkanoyl)amino groups. Particular examples of heterocycles include pyridyl, pyridonyl, pyrazinyl, pyrimidinyl, pyridazinyl, pyrrolyl, imidazolyl, pyrazolyl, triazolyl, tetrazolyl, furanyl, tetrahydrofuranyl, tetrahydropyranyl, dioxanyl, thienyl, oxazolyl, isoxazolyl, thiazolyl, oxadiazolyl, thiadiazolyl, indolyl, isoindolinyl, quinolyl, isoquinolyl, quinoxalyl, quinazolinyl and benzimidazolyl, each being optionally substituted as previously defined.

Details on a suitable assay system for identifying and/or studying an I:NEP are presented in the hereinafter in the section entitled NEP Assay.

Further examples of NEP inhibitors are disclosed and discussed in the following review articles:

- 15 Pathol. Biol., 46(3), 1998, 191.  
Current Pharm. Design, 2(5), 1996, 443.  
Biochem. Soc. Trans., 21(3), 1993, 678.  
Handbook Exp. Pharmacol., 104/1, 1993, 547.  
TiPS, 11, 1990, 245.
- 20 Pharmacol. Rev., 45(1), 1993, 87.  
Curr. Opin. Inves. Drugs, 2(11), 1993, 1175.  
Antihypertens. Drugs, (1997), 113.  
Chemtracts, (1997), 10(11), 804.  
Zinc Metalloproteases Health Dis. (1996), 105.
- 25 Cardiovasc. Drug Rev., (1996), 14(2), 166.  
Gen. Pharmacol., (1996), 27(4), 581.  
Cardiovasc. Drug Rev., (1994), 12(4), 271.  
Clin. Exp. Pharmacol. Physiol., (1995), 22(1), 63.  
Cardiovasc. Drug Rev., (1991), 9(3), 285.
- 30 Exp. Opin. Ther. Patents (1996), 6(11), 1147.

Yet, further examples of NEPi's are disclosed in the following documents:

EP-509442A  
US-192435  
US-4929641  
EP-599444B  
US-884664  
EP-544620A  
US-798684  
J. Med. Chem. 1993, 3821.  
Circulation 1993, 88(4), 1.  
EP-136883  
JP-85136554  
US-4722810  
Curr. Pharm. Design, 1996, 2, 443.  
EP-640594  
J. Med. Chem. 1993, 36(1), 87.  
EP-738711-A  
JP-270957  
CAS # 115406-23-0  
DE-19510566  
DE-19638020  
EP-830863  
JP-98101565  
EP-733642  
WO9614293  
JP-08245609  
JP-96245609  
WO9415908  
JP05092948  
WO-9309101  
WO-9109840  
EP-519738  
EP-690070  
J. Med. Chem. (1993), 36, 2420.  
JP-95157459  
Bioorg. Med. Chem. Letts., 1996, 6(1), 65.

Further I:NEPs are disclosed in the following documents:

EP-A-0274234

JP-88165353

Biochem.Biophys.Res. Comm.,1989, 164, 58

EP-629627-A

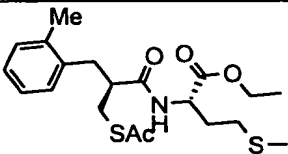
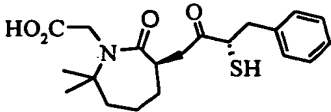
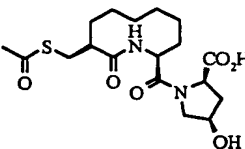
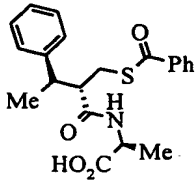
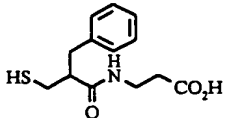
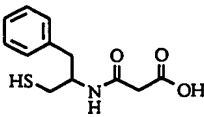
US-77978

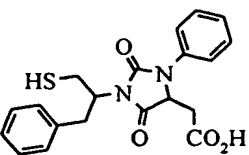
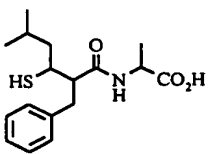
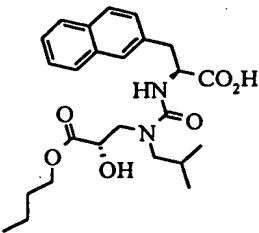
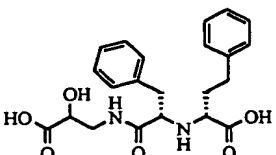
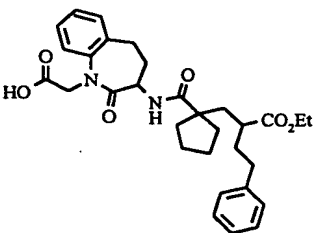
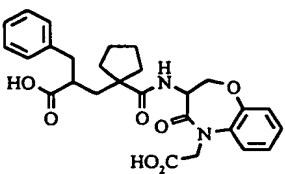
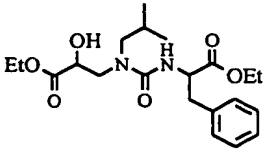
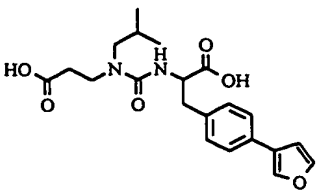
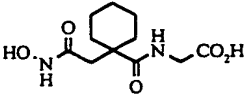
Perspect. Med. Chem. (1993), 45.

EP-358398-B

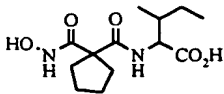
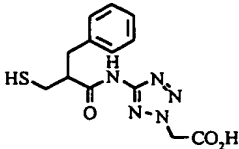
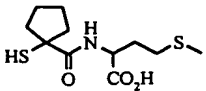
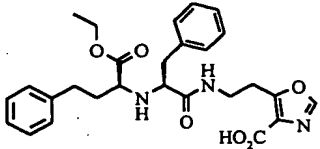
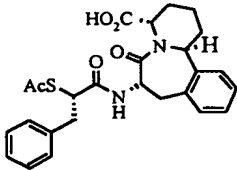
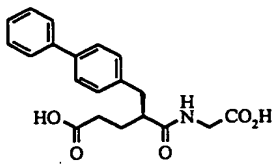
Further examples of I:NEPs are selected from the following structures:

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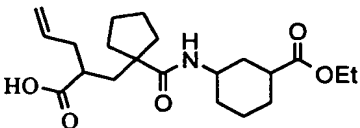
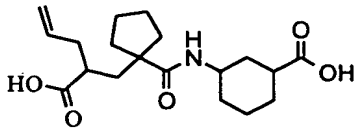
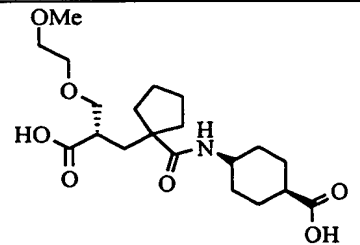
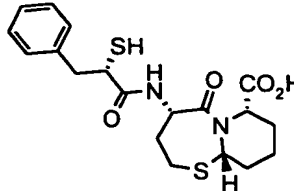
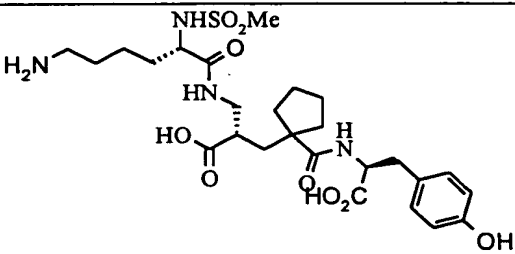
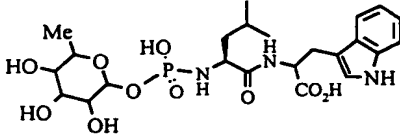
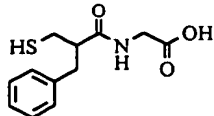
<u>Compound</u>	<u>Structure</u>	<u>Mode of Action</u> <u>References</u>
FXII		I:NEP EP-509442A US-192435 US-4929641
FXIII		I:NEP (also an ACE inhibitor) EP-599444B US-884664
FXIV		I:NEP EP-544620A US-798684 J. Med. Chem. 1993, 3821.
FXV		I:NEP (also an ACE inhibitor) Mixanpril Circulation 1993, <u>88</u> (4), 1.
FXVI		I:NEP EP-136883 JP-85136554 US-4722810
FXVII		I:NEP Retrothiorphan Curr. Pharm. Design, 1996, 2, 443.

FXVIII		I:NEP (also an ACE inhibitor) EP-640594
FXIX		I:NEP J. Med. Chem. 1993, 36(1), 87.
FXX		I:NEP (also an ACE inhibitor) EP-738711-A JP-270957
FXXI		I:NEP CAS # 115406-23-0
FXXII		I:NEP (also an ECE inhibitor) DE-19510566 DE-19638020 EP-830863 JP-98101565
FXXIII		I:NEP (also an ECE inhibitor) EP-733642
FXXIV		I:NEP WO96/14293
FXXV		I:NEP JP-08245609 JP-96245609
FXXVI		I:NEP WO9415908



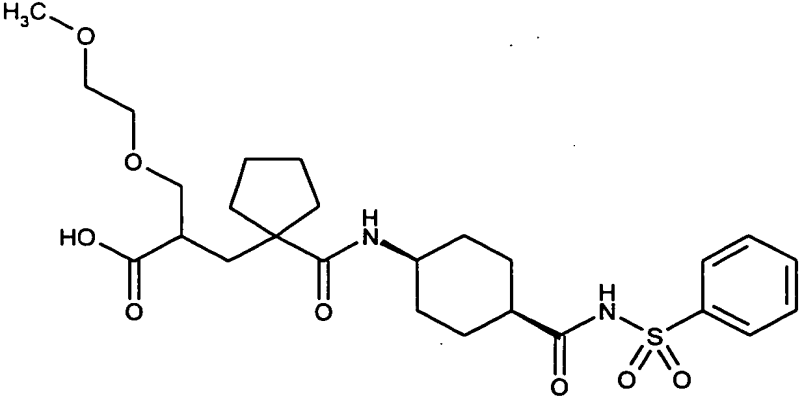
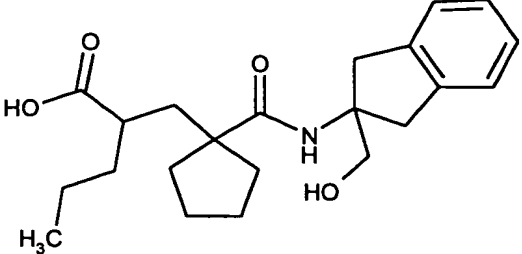
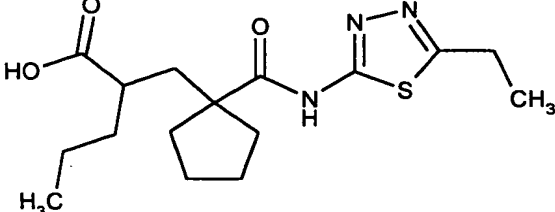
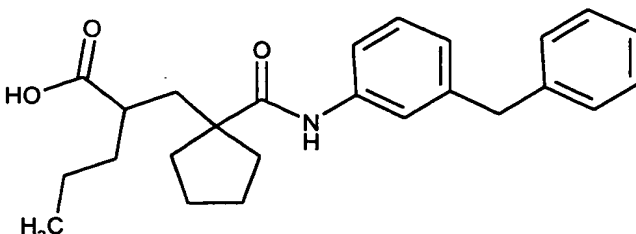
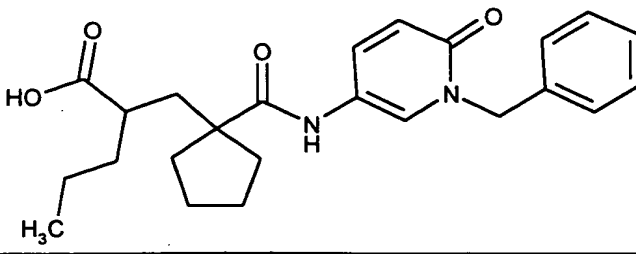
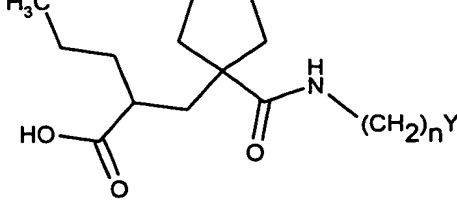
FXXVII		I:NEP JP05092948
FXXVIII		I:NEP WO-9309101
FXXIX		I:NEP WO-9109840
FXXXI		I:NEP EP-519738 EP-690070
FXXXII		I:NEP (also an ACE inhibitor) J. Med. Chem. (1993), 36, 2420.
FXXXIII		I:NEP JP-95157459 Bioorg. Med. Chem. Letts., 1996, 6(1), 65.

Preferred additional I:NEPs are selected from the following structures:

<u>Compound</u>	<u>Structure</u>	<u>Mode of Action</u> <u>References</u>
FV		I:NEP EP-A-0274234 (Example 300)
FVI		I:NEP EP-A-0274234 (Example 379)
FVII		I:NEP Candoxatrilat EP-274234 JP-88165353 Biochem.Biophys.Res. Comm.,1989, <u>164</u> , 58
FVIII		I:NEP Omapatrilat (also an inhibitor of ACE) EP-0629627-A US-77978
FIX		I:NEP Sampatrilat (also an inhibitor of ACE) Perspect. Med. Chem. (1993), 45. EP-0358398-B
FX		I:NEP Phosphoramidon (which is commercially available)
FXI		I:NEP Thiorphan (which is commercially available)

More preferred additional I:NEPs are selected from the following structures:

COMPOUND	STRUCTURE
F57	 <chem>COCC(=O)N1CCCC1C(=O)N2C(=O)C=CC(=CN2)CC3=CC=CC=C3</chem>
F58	 <chem>O=C(O)C(CC1=CC=CC=C1)CC2(CCCC2)C(=O)NCCCCN3CCCC3=O</chem>
F59	 <chem>O=C(O)C(CC1=CC=CC=C1)CC2(CCCC2)C(=O)N3C(CO)Cc4ccccc43</chem>
F60	 <chem>CC1=CN=C(S1)NC(=O)C2(CCCC2)C(=O)N3C(=O)C(CC4=CC=CC=C4)C(=O)O</chem>

F61	 <chem>COCCOCC1(CCCC1)C(=O)N[C@@H]2CCCC[C@H]2C(=O)NS(=O)(=O)c3ccccc3</chem>
F62	 <chem>CCCC(=O)O[C@H]1CCCC1C(=O)N[C@@H](CO)[C@H]2c3ccccc3CC2C(=O)O</chem>
F63	 <chem>CCCC(=O)O[C@H]1CCCC1C(=O)Nc2ncnc(s2)CC</chem>
F64	 <chem>CCCC(=O)O[C@H]1CCCC1C(=O)N[C@@H](Cc2ccccc2)[C@H](c3ccccc3)C</chem>
F65	 <chem>CCCC(=O)O[C@H]1CCCC1C(=O)Nc2ccc3c(c2)nc(Cc4ccccc4)c3=O</chem>
F66	 <chem>CCCC(=O)O[C@H]1CCCC1C(=O)NCCY</chem> (lc)

These compounds were prepared according to the teachings presented in the Experimental section (*infra*). These compounds were tested as agents and were found to be useful for enhancing the endogenous erectile process, and thereby being useful in the treatment of MED. Some of the experimental data concerning these compounds are presented in the Experimental section (*infra*).

Preferably, the NEP inhibitors for use in the treatment of MED according to the present invention have an  $IC_{50}$  at less than 100 nanomolar, more preferably, at less than 50 nanomolar, more preferably still less than 25 nanomolar and especially preferred less than or equal to 10 nanomolar.

Preferably the NEPi compounds for the use according to the present invention have at least about a 100 fold selectivity to the desired target, preferably at least about a 150 fold selectivity to the desired target, preferably at least about a 200 fold selectivity to the desired target, preferably at least about a 250 fold selectivity to the desired target, preferably at least about a 300 fold selectivity to the desired target, preferably at least about a 350 fold selectivity to the desired target.

More preferably the NEPi compounds for the use according to the present invention (and optionally the optional additional agent) has at least about a 400 fold selectivity to the desired target, preferably at least about a 500 fold selectivity to the desired target, preferably at least about a 600 fold selectivity to the desired target, preferably at least about a 700 fold selectivity to the desired target, preferably at least about a 800 fold selectivity to the desired target, preferably at least about a 900 fold selectivity to the desired target, preferably at least about a 1000 fold selectivity to the desired target.

Preferably, the NEP inhibitors for use in the treatment of MED according to the present invention have greater than 100-fold, more preferably greater than 300-fold and more preferably still greater than 3000-fold selectivity for NEP over either endothelin converting enzyme (ECE) or angiotensin converting enzyme (ACE).

More preferably still the NEP inhibitors for use in the treatment of MED according to the present invention have greater than 100-fold, more preferably greater than 300-fold and more preferably still greater than 3000-fold selectivity for NEP over both endothelin converting enzyme (ECE) and angiotensin converting enzyme (ACE).

Background teachings on NEP have been presented by Victor A. McKusick et al on <http://www3.ncbi.nlm.nih.gov/Omim/searchomim.htm>. The following information concerning NEP has been extracted from that source.

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"Common acute lymphocytic leukemia antigen is an important cell surface marker in the diagnosis of human acute lymphocytic leukemia (ALL). It is present on leukemic cells of pre-B phenotype, which represent 85% of cases of ALL. CALLA is not restricted to leukemic cells, however, and is found on a variety of normal tissues. CALLA is a glycoprotein that is particularly abundant in kidney, where it is present on the brush border of proximal tubules and on glomerular epithelium. Letarte et al. (1988) cloned a cDNA coding for CALLA and showed that the amino acid sequence deduced from the cDNA sequence is identical to that of human membrane-associated neutral endopeptidase (NEP; EC 3.4.24.11 ), also known as enkephalinase. NEP cleaves peptides at the amino side of hydrophobic residues and inactivates several peptide hormones including glucagon, enkephalins, substance P, neurotensin, oxytocin, and bradykinin. By cDNA transfection analysis, Shipp et al. (1989) confirmed that CALLA is a functional neutral endopeptidase of the type that has previously been called enkephalinase. Barker et al. (1989) demonstrated that the CALLA gene, which encodes a 100-kD type II transmembrane glycoprotein, exists in a single copy of greater than 45 kb which is not rearranged in malignancies expressing cell surface CALLA. The gene was located to human chromosome 3 by study of somatic cell hybrids and in situ hybridization regionalized the location to 3q21-q27. Tran-Paterson et al. (1989) also assigned the gene to chromosome 3 by Southern blot analysis of DNA from human-rodent somatic cell hybrids. D'Adamio et al. (1989) demonstrated that the CALLA gene spans more than 80 kb and is composed of 24 exons."

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### PDE5 Inhibitors

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As detailed hereinbefore according to a further embodiment of the present invention there is provided use of a pharmaceutical composition comprising an NEPi and an I:PDE<sub>cGMP</sub> for use in the treatment of MED. More particularly the present invention provides use of a pharmaceutical composition comprising an NEPi and an I:PDE<sub>5cGMP</sub> in the manufacture of a medicament for the treatment of MED.

Suitable PDE5i's for use in the pharmaceutical compositions according to the present invention are the cGMP PDE5i's hereinafter detailed. Particularly preferred for use herein are potent and selective cGMP PDE5i's.

- 5 Suitable cGMP PDE5 inhibitors for the use according to the present invention include:

the pyrazolo [4,3-d]pyrimidin-7-ones disclosed in EP-A-0463756; the pyrazolo [4,3-d]pyrimidin-7-ones disclosed in EP-A-0526004; the pyrazolo [4,3-d]pyrimidin-7-ones  
10 disclosed in published international patent application WO 93/06104; the isomeric pyrazolo [3,4-d]pyrimidin-4-ones disclosed in published international patent application WO 93/07149; the quinazolin-4-ones disclosed in published international patent application WO 93/12095; the pyrido [3,2-d]pyrimidin-4-ones disclosed in published international patent application WO 94/05661; the purin-6-ones disclosed  
15 in published international patent application WO 94/00453; the pyrazolo [4,3-d]pyrimidin-7-ones disclosed in published international patent application WO 98/49166; the pyrazolo [4,3-d]pyrimidin-7-ones disclosed in published international patent application WO 99/54333; the pyrazolo [4,3-d]pyrimidin-4-ones disclosed in EP-A-0995751; the pyrazolo [4,3-d]pyrimidin-7-ones disclosed in published  
20 international patent application WO 00/24745; the pyrazolo [4,3-d]pyrimidin-4-ones disclosed in EP-A-0995750; the compounds disclosed in published international application WO95/19978; the compounds disclosed in published international application WO 99/24433 and the compounds disclosed in published international application WO 93/07124.

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It is to be understood that the contents of the above published patent applications, and in particular the general formulae and exemplified compounds therein are incorporated herein in their entirety by reference thereto.

- 30 Preferred type V phosphodiesterase inhibitors for the use according to the present invention include:

5-[2-ethoxy-5-(4-methyl-1-piperazinylsulphonyl)phenyl]-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (sildenafil) also known as 1-[[3-(6,7-  
35 dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulphonyl]-4-methylpiperazine (see EP-A-0463756);

5-(2-ethoxy-5-morpholinoacetylphenyl)-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see EP-A-0526004);

5 3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-n-propoxyphenyl]-2-(pyridin-2-yl)methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see WO98/49166);

3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-(2-methoxyethoxy)pyridin-3-yl]-2-(pyridin-2-yl)methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see WO99/54333);

10

(+)-3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-(2-methoxy-1(R)-methylethoxy)pyridin-3-yl]-2-methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one, also known as 3-ethyl-5-{5-[4-ethylpiperazin-1-ylsulphonyl]-2-[(1R)-2-methoxy-1-methylethyl]oxy}pyridin-3-yl]-2-methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see WO99/54333);

15

5-[2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-[2-methoxyethyl]-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one, also known as 1-{6-ethoxy-5-[3-ethyl-6,7-dihydro-2-(2-methoxyethyl)-7-oxo-2H-pyrazolo[4,3-d]pyrimidin-5-yl]-3-pyridylsulphonyl}-4-ethylpiperazine (see PDE5 Example 1 hereinafter);

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5-[2-iso-Butoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-(1-methylpiperidin-4-yl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see PDE5 Example 2 hereinafter);

25

5-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-phenyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see PDE5 Example 3 hereinafter);

30

5-(5-Acetyl-2-propoxy-3-pyridinyl)-3-ethyl-2-(1-isopropyl-3-azetidiny)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see PDE5 Example 4 hereinafter);

5-(5-Acetyl-2-butoxy-3-pyridinyl)-3-ethyl-2-(1-ethyl-3-azetidiny)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (see PDE5 Example 5 hereinafter);

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(6R,12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylenedioxyphenyl)-pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione (IC-351), i.e. the compound of



examples 78 and 95 of published international application WO95/19978, as well as the compound of examples 1, 3, 7 and 8;

2-[2-ethoxy-5-(4-ethyl-piperazin-1-yl-1-sulphonyl)-phenyl]-5-methyl-7-propyl-3H-imidazo[5,1-f][1,2,4]triazin-4-one (vardenafil) also known as 1-[[3-(3,4-dihydro-5-methyl-4-oxo-7-propylimidazo[5,1-f]-as-triazin-2-yl)-4-ethoxyphenyl]sulphonyl]-4-ethylpiperazine, i.e. the compound of examples 20, 19, 337 and 336 of published international application WO99/24433; and

the compound of example 11 of published international application WO93/07124 (EISAI); and

compounds 3 and 14 from Rotella D P, *J. Med. Chem.*, 2000, 43, 1257.

Still other type cGMP PDE5 inhibitors useful in conjunction with the present invention include: 4-bromo-5-(pyridylmethylamino)-6-[3-(4-chlorophenyl)-propoxy]-3(2H)pyridazinone; 1-[4-[(1,3-benzodioxol-5-ylmethyl)amino]-6-chloro-2-quinazolinyl]-4-piperidine-carboxylic acid, monosodium salt; (+)-cis-5,6a,7,9,9,9a-hexahydro-2-[4-(trifluoromethyl)-phenylmethyl-5-methyl-cyclopent-4,5]imidazo[2,1-b]purin-4(3H)one; furazlocillin; cis-2-hexyl-5-methyl-3,4,5,6a,7,8,9,9a-octahydrocyclopent[4,5]-imidazo[2,1-b]purin-4-one; 3-acetyl-1-(2-chlorobenzyl)-2-propylindole-6-carboxylate; 3-acetyl-1-(2-chlorobenzyl)-2-propylindole-6-carboxylate; 4-bromo-5-(3-pyridylmethylamino)-6-(3-(4-chlorophenyl)propoxy)-3(2H)pyridazinone; 1-methyl-5(5-morpholinoacetyl-2-n-propoxyphenyl)-3-n-propyl-1,6-dihydro-7H-pyrazolo(4,3-d)pyrimidin-7-one; 1-[4-[(1,3-benzodioxol-5-ylmethyl)amino]-6-chloro-2-quinazolinyl]-4-piperidinecarboxylic acid, monosodium salt; Pharmaprojects No. 4516 (Glaxo Wellcome); Pharmaprojects No. 5051 (Bayer); Pharmaprojects No. 5064 (Kyowa Hakko; see WO 96/26940); Pharmaprojects No. 5069 (Schering Plough); GF-196960 (Glaxo Wellcome); E-8010 and E-4010 (Eisai); Bay-38-3045 & 38-9456 (Bayer) and Sch-51866.

The suitability of any particular cGMP PDE5 inhibitor can be readily determined by evaluation of its potency and selectivity using literature methods followed by evaluation of its toxicity, absorption, metabolism, pharmacokinetics, etc in accordance with standard pharmaceutical practice.

Preferably, the cGMP PDE5 inhibitors have an  $IC_{50}$  at less than 100 nanomolar, more preferably, at less than 50 nanomolar, more preferably still at less than 10 nanomolar.

- 5  $IC_{50}$  values for the cGMP PDE5 inhibitors may be determined using established literature methodology, for example as described in EP0463756-B1 and EP0526004-A1 and as detailed in the Test Methods Section hereinafter.

- 10 Preferably the cGMP PDE5 inhibitors used in the pharmaceutical compositions according to the present invention are selective for the PDE5 enzyme. Preferably they are selective over PDE3, more preferably over PDE3 and PDE4. Preferably, the cGMP PDE5 inhibitors of the invention have a selectivity ratio greater than 100 more preferably greater than 300, over PDE3 and more preferably over PDE3 and PDE4.

- 15 Selectivity ratios may readily be determined by the skilled person.  $IC_{50}$  values for the PDE3 and PDE4 enzyme may be determined using established literature methodology, see S A Ballard *et al*, Journal of Urology, 1998, vol. 159, pages 2164-2171 and as detailed herein after.

- 20 Highly preferred for use in the pharmaceutical compositions herein are potent and selective PDE5 inhibitors.

- As discussed hereinbefore the NEPi (and PDE5i where applicable) for use in the treatment of MED in accordance with the present invention may be administered in  
25 conjunction with a further pharmaceutically active agent. Here the co-administration need not be done at the same time, let alone by the same route. An example of a co-administration composition could be a composition that comprises an NEPi and an additional agent.

- 30 For some applications, the additional agent is an inhibitor – i.e. it is capable of exhibiting an inhibitory function.

For some applications, preferably the additional agent is a NPYi (sometimes written as I:NPY).

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For some applications, preferably the additional agent is an NPYi Y1 or NPYi Y2 or NPYi Y5, more preferably the agent is an NPYi Y1.

For some applications, preferably the additional agent is a selective NPYi.

For some applications, preferably the additional agent is an NPYi Y1.

5

For some applications, preferably the additional agent is a selective NPYi Y1.

## TREATMENT

10 It is to be appreciated that all references herein to treatment include one or more of curative, palliative and prophylactic treatment. Preferably, the term treatment includes at least curative treatment and/or palliative treatment.

## 15 SEXUAL STIMULATION

The present invention also encompasses use as defined hereinbefore via administration of a NEPi (and an PDE5i where applicable) before and/or during sexual stimulation. Here the term "sexual stimulation" may be synonymous with the  
20 term "sexual arousal". This aspect of the present invention is advantageous because it provides systemic selectivity. The natural cascade only occurs at the genitalia and not in other locations – e.g. in the heart etc. Hence, it would be possible to achieve a selective effect on the genitalia via the MED treatment according to the present invention.

25

Thus, according to the present invention it is highly desirable that there is a sexual stimulation step at some stage. We have found that this step can provide systemic selectivity. Here, "sexual stimulation" may be one or more of a visual stimulation, a physical stimulation, an auditory stimulation, or a thought stimulation.

30

## AGENT

35 Agents for use in the treatment of MED according to of the present invention may be any suitable agent that can act as a NEPi and, where appropriate as a PDE5i.

Such agents (i.e. the agents as defined above and/or the additional agents as defined hereinbefore) can be an amino acid sequence or a chemical derivative thereof. The substance may even be an organic compound or other chemical. The agent may even be a nucleotide sequence - which may be a sense sequence or an anti-sense sequence. The agent may even be an antibody.

Thus, the term "agent" includes, but is not limited to, a compound which may be obtainable from or produced by any suitable source, whether natural or not.

The agent may be designed or obtained from a library of compounds which may comprise peptides, as well as other compounds, such as small organic molecules, such as lead compounds.

By way of example, the agent may be a natural substance, a biological macromolecule, or an extract made from biological materials such as bacteria, fungi, or animal (particularly mammalian) cells or tissues, an organic or an inorganic molecule, a synthetic agent, a semi-synthetic agent, a structural or functional mimetic, a peptide, a peptidomimetics, a derivatised agent, a peptide cleaved from a whole protein, or a peptides synthesised synthetically (such as, by way of example, either using a peptide synthesizer or by recombinant techniques or combinations thereof, a recombinant agent, an antibody, a natural or a non-natural agent, a fusion protein or equivalent thereof and mutants, derivatives or combinations thereof.

As used herein, the term "agent" may be a single entity or it may be a combination of agents.

If the agent is an organic compound then for some applications - such as if the agent is a NEPi - that organic compound may typically comprise an amide group (i.e. -N(H)-C(O)- or even -C(O)-N(H)-) and one or more hydrocarbyl groups. Here, the term "hydrocarbyl group" means a group comprising at least C and H and may optionally comprise one or more other suitable substituents. Examples of such substituents may include halo-, alkoxy-, nitro-, an alkyl group, a cyclic group etc. In addition to the possibility of the substituents being a cyclic group, a combination of substituents may form a cyclic group. If the hydrocarbyl group comprises more than one C then those carbons need not necessarily be linked to each other. For example, at least two of the carbons may be linked via a suitable element or group. Thus, the hydrocarbyl group may contain hetero atoms. Suitable hetero atoms will

be apparent to those skilled in the art and include, for instance, sulphur, nitrogen and oxygen. For some applications, preferably the agent comprises at least one cyclic group. For some applications, preferably the agent comprises at least one cyclic group linked to another hydrocarbonyl group via an amide bond. Examples of such compounds are presented in the Additional Compound Examples section herein.

If the agent is an organic compound then for some applications - such as if the agent is an PDE5i - that organic compound may typically comprise two or more linked hydrocarbonyl groups. For some applications, preferably the agent comprises at least two cyclic groups - wherein one of which cyclic groups may be a fused cyclic ring structure. For some applications, preferably at least one of the cyclic groups is a heterocyclic group. For some applications, preferably the heterocyclic group comprises at least one N in the ring. Examples of such compounds are presented in the PDE5 Examples section herein.

If the agent is an organic compound then for some applications - such as if the agent is an I:NPY - that organic compound may typically comprise two or more linked hydrocarbonyl groups. For some applications, preferably the agent comprises at least two cyclic groups - optionally wherein one of which cyclic groups may be a fused cyclic ring structure. For some applications, at least one of the cyclic groups is a heterocyclic group. For some applications, preferably the heterocyclic group comprises at least one N in the ring. Examples of such compounds are presented in the Additional Examples section herein.

The agent may contain halo groups. Here, "halo" means fluoro, chloro, bromo or iodo.

The agent may contain one or more of alkyl, alkoxy, alkenyl, alkylene and alkenylene groups - which may be unbranched- or branched-chain.

The agent may be in the form of a pharmaceutically acceptable salt - such as an acid addition salt or a base salt - or a solvate thereof, including a hydrate thereof. For a review on suitable salts see Berge *et al*, J. Pharm. Sci., 1977, 66, 1-19.

Suitable acid addition salts are formed from acids which form non-toxic salts and examples are the hydrochloride, hydrobromide, hydroiodide, sulphate, bisulphate, nitrate, phosphate, hydrogen phosphate, acetate, maleate, fumarate, lactate, tartrate,

citrate, gluconate, succinate, saccharate, benzoate, methanesulphonate, ethanesulphonate, benzenesulphonate, p-toluenesulphonate and pamoate salts.

5 Sutable base salts are formed from bases which form non-toxic salts and examples are the sodium, potassium, aluminium, calcium, magnesium, zinc and diethanolamine salts.

10 A pharmaceutically acceptable salt of an agent as defined hereinbefore may be readily prepared by mixing together solutions of the agent and the desired acid or base, as appropriate. The salt may precipitate from solution and be collected by filtration or may be recovered by evaporation of the solvent.

The agent may exist in polymorphic form.

15 The agent may contain one or more asymmetric carbon atoms and therefore exists in two or more stereoisomeric forms. Where an agent contains an alkenyl or alkenylene group, cis (E) and trans (Z) isomerism may also occur. The present invention includes the individual stereoisomers of the agent and, where appropriate, the individual tautomeric forms thereof, together with mixtures thereof.

20 Separation of diastereoisomers or cis and trans isomers may be achieved by conventional techniques, e.g. by fractional crystallisation, chromatography or H.P.L.C. of a stereoisomeric mixture of the agent or a suitable salt or derivative thereof. An individual enantiomer of the agent may also be prepared from a  
25 corresponding optically pure intermediate or by resolution, such as by H.P.L.C. of the corresponding racemate using a suitable chiral support or by fractional crystallisation of the diastereoisomeric salts formed by reaction of the corresponding racemate with a suitable optically active acid or base, as appropriate.

30 The present invention also includes all suitable isotopic variations of the agent or a pharmaceutically acceptable salt thereof. An isotopic variation of an agent of the present invention or a pharmaceutically acceptable salt thereof is defined as one in which at least one atom is replaced by an atom having the same atomic number but an atomic mass different from the atomic mass usually found in nature. Examples of  
35 isotopes that can be incorporated into the agent and pharmaceutically acceptable salts thereof include isotopes of hydrogen, carbon, nitrogen, oxygen, phosphorus, sulphur, fluorine and chlorine such as  $^2\text{H}$ ,  $^3\text{H}$ ,  $^{13}\text{C}$ ,  $^{14}\text{C}$ ,  $^{15}\text{N}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ,  $^{31}\text{P}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^{18}\text{F}$

and  $^{36}\text{Cl}$ , respectively. Certain isotopic variations of the agent and pharmaceutically acceptable salts thereof, for example, those in which a radioactive isotope such as  $^3\text{H}$  or  $^{14}\text{C}$  is incorporated, are useful in drug and/or substrate tissue distribution studies. Tritiated, i.e.,  $^3\text{H}$ , and carbon-14, i.e.,  $^{14}\text{C}$ , isotopes are particularly preferred for their ease of preparation and detectability. Further, substitution with isotopes such as deuterium, i.e.,  $^2\text{H}$ , may afford certain therapeutic advantages resulting from greater metabolic stability, for example, increased *in vivo* half-life or reduced dosage requirements and hence may be preferred in some circumstances. Isotopic variations of the agent and pharmaceutically acceptable salts thereof can generally be prepared by conventional procedures using appropriate isotopic variations of suitable reagents.

It will be appreciated by those skilled in the art that the agent may be derived from a prodrug. Examples of prodrugs include entities that have certain protected group(s) and which may not possess pharmacological activity as such, but may, in certain instances, be administered (such as orally or parenterally) and thereafter metabolised in the body to form the agent which are pharmacologically active.

It will be further appreciated that certain moieties known as "pro-moieties", for example as described in "Design of Prodrugs" by H. Bundgaard, Elsevier, 1985 (the disclosed of which is hereby incorporated by reference), may be placed on appropriate functionalities of the agents. Such prodrugs are also included within the scope of the invention.

The term inhibitor as used herein in relation to the NEPi and PDE5i compounds is to be regarded as being interchangeable with the term antagonist. Further the phrase, enhancing the endogenous erectile process, is to be regarded as being interchangeable with the phrase upregulation of the endogenous erectile process.

For some applications (such as a topical application), the agent may also display an ACE (angiotensin converting enzyme) inhibitory action. An ACE assay is presented in the Experimental Section herein. For some applications (such as with particular patient types), such agents (i.e. those that also display ACE inhibitory action) may not be suitable for oral administration.

For some applications, the agent may also display an ECE (endothelium converting enzyme) inhibitory action. ECE assays are well known in the art.

## PHARMACEUTICAL COMBINATIONS

As discussed hereinbefore treatment of MED according to the present invention may be achieved via a combination of a NEPi with one or more other additional pharmaceutically active agents, such as a nitric oxide donor, or a nitric oxide precursor eg L-arginine or inhibitors of arginase) and/or a centrally acting pharmaceutical (e.g. a dopamine receptor agonist such as apomorphine or selective dopamine D2 receptor agonists such as PNU-95666 or a melanocortin receptor agonist, such as melanotan II). Teachings on the use of apomorphine as a pharmaceutical may be found in US-A-5945117. In that particular document, apomorphine is delivered sub-lingually. In addition, or in the alternative, the agent may be used in combination with one or more of: one or more of a nitric oxide donor (eg NMI-921), one or more of a dopamine receptor agonist (eg apomorphine, Uprima, Ixsene), one or more of a heterocyclic amine such as generically and specifically disclosed in WO 00/40226, in particular example numbers 7, 8 and 9, one or more of a melanocortin receptor agonist (eg Melanotan II or PT14), one or more of a potassium channel opener (eg a  $K_{ATP}$  channel opener (eg minoxidil, nicorandil) and/or a calcium activated potassium channel opener (eg BMS-204352), one or more of an  $\alpha$ 1-adrenoceptor antagonist (eg phentolamine, Vasomax), one or more of a VIP receptor agonist or a VIP analogue (eg Ro-125-1553) or a VIP fragment, one or more of a  $\alpha$ -adrenoceptor antagonist with VIP combination (eg Invicorp, Avipitadil), one or more of a  $\alpha$ 2-adrenoceptor antagonist (eg yohimbine), one or more of a testosterone replacement agent (inc DHEA (dehydroandrostendione), testosterone (Tostrelle) or a testosterone implant (Organon)), one or more of a testosterone/oestradiol agent one or more of an estrogen agonists eg Lasofoxifene, one or more of a serotonin receptor agonist or antagonist (eg 5HT1A, 5HT2C, 5HT2A and 5HT3 receptor agonists and antagonists; as described in WO2000/28993), one or more of a prostanoid receptor agonist (eg Muse, alprostadiol, misoprostol), one or more of a purinergic receptor agonist (especially P2Y2 and P2Y4) one or more antidepressant agents (eg bupropion (Wellbutrin), mirtazapine, nefazodone).

If a combination of additional active agents are administered, then they may be administered simultaneously, separately or sequentially with or to the NEPi as detailed hereinbefore.



## Pharmaceutical Formulations

The compounds of the invention, their pharmaceutically acceptable salts, and pharmaceutically acceptable solvates of either entity can be administered alone but, in human therapy will generally be administered in admixture with a suitable pharmaceutical excipient diluent or carrier selected with regard to the intended route of administration and standard pharmaceutical practice.

For example, the compounds of the invention, or salts or solvates thereof can be administered orally, buccally or sublingually in the form of tablets, capsules (including soft gel capsules), ovules, elixirs, solutions or suspensions, which may contain flavouring or colouring agents, for immediate-, delayed-, modified-, or controlled-release such as sustained-, dual-, or pulsatile delivery applications. The compounds of the invention may also be administered via intracavernosal injection. The compounds of the invention may also be administered via fast dispersing or fast dissolving dosages forms or in the form of a high energy dispersion or as coated particles. Suitable pharmaceutical formulations of the compounds of the invention may be in coated or un-coated form as desired.

Such tablets may contain excipients such as microcrystalline cellulose, lactose, sodium citrate, calcium carbonate, dibasic calcium phosphate, glycine and starch (preferably corn, potato or tapioca starch), disintegrants such as sodium starch glycollate, croscarmellose sodium and certain complex silicates, and granulation binders such as polyvinylpyrrolidone, hydroxypropylmethyl cellulose (HPMC), hydroxypropylcellulose (HPC), sucrose, gelatin and acacia. Additionally, lubricating agents such as magnesium stearate, stearic acid, glyceryl behenate and talc may be included.

Solid compositions of a similar type may also be employed as fillers in gelatin capsules. Preferred excipients in this regard include lactose, starch, a cellulose, milk sugar or high molecular weight polyethylene glycols. For aqueous suspensions and/or elixirs, the compounds of the invention may be combined with various sweetening or flavouring agents, colouring matter or dyes, with emulsifying and/or suspending agents and with diluents such as water, ethanol, propylene glycol and glycerin, and combinations thereof.

Modified release and pulsatile release dosage forms may contain excipients such as those detailed for immediate release dosage forms together with additional excipients that act as release rate modifiers, these being coated on and/or included in the body of the device. Release rate modifiers include, but are not exclusively limited to, hydroxypropylmethyl cellulose, methyl cellulose, sodium carboxymethylcellulose, ethyl cellulose, cellulose acetate, polyethylene oxide, Xanthan gum, Carbomer, ammonio methacrylate copolymer, hydrogenated castor oil, carnauba wax, paraffin wax, cellulose acetate phthalate, hydroxypropylmethyl cellulose phthalate, methacrylic acid copolymer and mixtures thereof. Modified release and pulsatile release dosage forms may contain one or a combination of release rate modifying excipients. Release rate modifying excipients maybe present both within the dosage form i.e. within the matrix, and/or on the dosage form i.e. upon the surface or coating.

Fast dispersing or dissolving dosage formulations (FDDFs) may contain the following ingredients: aspartame, acesulfame potassium, citric acid, croscarmellose sodium, crospovidone, diascorbic acid, ethyl acrylate, ethyl cellulose, gelatin, hydroxypropylmethyl cellulose, magnesium stearate, mannitol, methyl methacrylate, mint flavouring, polyethylene glycol, fumed silica, silicon dioxide, sodium starch glycolate, sodium stearyl fumarate, sorbitol, xylitol. The terms dispersing or dissolving as used herein to describe FDDFs are dependent upon the solubility of the drug substance used i.e. where the drug substance is insoluble a fast dispersing dosage form can be prepared and where the drug substance is soluble a fast dissolving dosage form can be prepared.

The compounds of the invention can also be administered parenterally, for example, intracaverosally, intravenously, intra-arterially, intraperitoneally, intrathecally, intraventricularly, intraurethrally intrasternally, intracranially, intramuscularly or subcutaneously, or they may be administered by infusion or needless injection techniques. For such parenteral administration they are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well-known to those skilled in the art.

For oral and parenteral administration to human patients, the daily dosage level of the compounds of the invention or salts or solvates thereof will usually be from 10 to 500 mg (in single or divided doses).

- 5 Thus, for example, tablets or capsules of the compounds of the invention or salts or solvates thereof may contain from 5 mg to 250 mg of active compound for administration singly or two or more at a time, as appropriate. The physician in any event will determine the actual dosage which will be most suitable for any individual patient and it will vary with the age, weight and response of the particular patient.
- 10 The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited and such are within the scope of this invention. The skilled person will also appreciate that, for in the treatment of MED according to the present invention, the NEPi (and where appropriate PDE5i or additional agents(s)) compounds may be taken as a single
- 15 dose on an "as required" basis (i.e. as needed or desired).

#### Example Tablet Formulation

- In general a tablet formulation could typically contain between about 0.01mg and
- 20 500mg of compound (or a salt thereof) whilst tablet fill weights may range from 50mg to 1000mg. An example formulation for a 10mg tablet is illustrated:

<u>Ingredient</u>	<u>%w/w</u>
Free acid, Free base or Salt of Compound	10.000*
25 Lactose	64.125
Starch	21.375
Croscarmellose Sodium	3.000
Magnesium Stearate	1.500

- 30 \* This quantity is typically adjusted in accordance with drug activity.

The tablets are manufactured by a standard process, for example, direct compression or a wet or dry granulation process. The tablet cores may be coated with appropriate overcoats.

The compounds / compositions can also be administered intranasally or by inhalation and are conveniently delivered in the form of a dry powder inhaler or an aerosol spray presentation from a pressurised container, pump, spray or nebuliser with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, 5 dichlorotetrafluoroethane, a hydrofluoroalkane such as 1,1,1,2-tetrafluoroethane (HFA 134A [trade mark] or 1,1,1,2,3,3,3-heptafluoropropane (HFA 227EA [trade mark])), carbon dioxide or other suitable gas. In the case of a pressurised aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. The pressurised container, pump, spray or nebuliser may contain a solution 10 or suspension of the active compound, e.g. using a mixture of ethanol and the propellant as the solvent, which may additionally contain a lubricant, e.g. sorbitan trioleate. Capsules and cartridges (made, for example, from gelatin) for use in an inhaler or insufflator may be formulated to contain a powder mix of a compound of the invention and a suitable powder base such as lactose or starch.

15 Aerosol or dry powder formulations are preferably arranged so that each metered dose or "puff" contains from 1 to 50 mg of a compound of the invention for delivery to the patient. The overall daily dose with an aerosol will be in the range of from 1 to 50 mg which may be administered in a single dose or, more usually, in divided doses 20 throughout the day.

The compounds also be formulated for delivery via an atomiser. Formulations for atomiser devices may contain the following ingredients as solubilisers, emulsifiers or suspending agents: water, ethanol, glycerol, propylene glycol, low molecular weight 25 polyethylene glycols, sodium chloride, fluorocarbons, polyethylene glycol ethers, sorbitan trioleate, oleic acid.

Alternatively, the compounds or salts or solvates thereof can be administered in the form of a suppository, or they may be applied topically in the form of a gel, hydrogel, 30 lotion, solution, cream, ointment or dusting powder. The compounds of the invention or salts or solvates thereof may also be dermally administered. The compounds of the invention or salts or solvates thereof may also be transdermally administered, for example, by the use of a skin patch. They may also be administered by the ocular, pulmonary or rectal routes.

35 For ophthalmic use, the compounds can be formulated as micronised suspensions in isotonic, pH adjusted, sterile saline, or, preferably, as solutions in isotonic, pH

adjusted, sterile saline, optionally in combination with a preservative such as a benzylalkonium chloride. Alternatively, they may be formulated in an ointment such as petrolatum.

5 For application topically to the skin, the compounds or salts or solvates thereof can be formulated as a suitable ointment containing the active compound suspended or dissolved in, for example, a mixture with one or more of the following: mineral oil, liquid petrolatum, white petrolatum, propylene glycol, polyoxyethylene polyoxypropylene compound, emulsifying wax and water. Alternatively, they can be  
10 formulated as a suitable lotion or cream, suspended or dissolved in, for example, a mixture of one or more of the following: mineral oil, sorbitan monostearate, a polyethylene glycol, liquid paraffin, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water.

15 The compounds may also be used in combination with a cyclodextrin. Cyclodextrins are known to form inclusion and non-inclusion complexes with drug molecules. Formation of a drug-cyclodextrin complex may modify the solubility, dissolution rate, bioavailability and/or stability property of a drug molecule. Drug-cyclodextrin complexes are generally useful for most dosage forms and administration routes. As  
20 an alternative to direct complexation with the drug the cyclodextrin may be used as an auxiliary additive, e.g. as a carrier, diluent or solubiliser. Alpha-, beta- and gamma-cyclodextrins are most commonly used and suitable examples are described in WO-A-91/11172, WO-A-94/02518 and WO-A-98/55148.

25 Generally, in humans, oral administration of the is the preferred route, being the most convenient in MED, avoiding the well-known disadvantages associated with intracavernosal (i.c.) administration. A preferred oral dosing regimen in MED for a typical man is from about 25mg to 500 mg of pharmaceutical composition when required. Where the composition comprises the combination of a NEPi and a PDE5I  
30 then from 25mg to 250mg of each compound may be present. In circumstances where the recipient suffers from a swallowing disorder or from impairment of drug absorption after oral administration, the drug may be administered parenterally, sublingually or buccally.

35 K<sub>i</sub> VALUES

For some applications, preferably the agent of the present invention (and optionally the optional additional agent) has a  $K_i$  value of less than about 100 nM, preferably less than about 75 nM, preferably less than about 50 nM, preferably less than about 25 nM, preferably less than about 20 nM, preferably less than about 15 nM, preferably less than about 10 nM, preferably less than about 5 nM.

### PHARMACOKINETICS

For some embodiments of the present invention, preferably the NEPi agents for use in the treatment of MED according to the present invention (and optionally the optional additional agent) have a log D of -2 to +4, more preferably -1 to +2. The log D can be determined by standard procedures known in the art such as described in J. Pharm. Pharmacol. 1990, 42:144.

In addition, or in the alternative to the above, for some embodiments preferably the NEPi agents (and optionally the PDE5i and/or optional additional agent(s)) have a caco-2 flux of greater than  $2 \times 10^{-6} \text{cms}^{-1}$ , more preferably greater than  $5 \times 10^{-6} \text{cms}^{-1}$ . The caco flux value can be determined by standard procedures known in the art such as described in J. Pharm. Sci 79, 7, p595-600 (1990), and Pharm. Res. vol 14, no. 6 (1997).

### CHEMICAL SYNTHESIS METHODS

Typically the NEPi and PDE5i compounds suitable for the use according to the present invention will be prepared by chemical synthesis techniques.

The agent or target or variants, homologues, derivatives, fragments or mimetics thereof may be produced using chemical methods to synthesize the agent in whole or in part. For example, peptides can be synthesized by solid phase techniques, cleaved from the resin, and purified by preparative high performance liquid chromatography (e.g., Creighton (1983) Proteins Structures And Molecular Principles, WH Freeman and Co, New York NY). The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure; Creighton, *supra*).

Direct synthesis of the agent or variants, homologues, derivatives, fragments or mimetics thereof can be performed using various solid-phase techniques (Roberge JY *et al* (1995) Science 269: 202-204) and automated synthesis may be achieved, for example, using the ABI 431 A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer. Additionally, the amino acid sequences comprising the agent or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with a sequence from other subunits, or any part thereof, to produce a variant agent or target, such as, for example, a variant NEP.

In an alternative embodiment of the invention, the coding sequence of the agent target or variants, homologues, derivatives, fragments or mimetics thereof may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al* (1980) Nuc Acids Res Symp Ser 215-23, Horn T *et al* (1980) Nuc Acids Res Symp Ser 225-232).

#### MIMETIC

As used herein, the term "mimetic" relates to any chemical which includes, but is not limited to, a peptide, polypeptide, antibody or other organic chemical which has the same qualitative activity or effect as a reference agent to a target.

#### CHEMICAL DERIVATIVE

The term "derivative" or "derivatised" as used herein includes chemical modification of an agent. Illustrative of such chemical modifications would be replacement of hydrogen by a halo group, an alkyl group, an acyl group or an amino group.

#### CHEMICAL MODIFICATION

In one embodiment of the present invention, the agent may be a chemically modified agent.

The chemical modification of an agent may either enhance or reduce hydrogen bonding interaction, charge interaction, hydrophobic interaction, Van Der Waals interaction or dipole interaction between the agent and the target.

In one aspect, the identified agent may act as a model (for example, a template) for the development of other compounds.

#### RECOMBINANT METHODS

5

Typically the target for use in the assay of the present invention may be prepared by recombinant DNA techniques.

#### 10 AMINO ACID SEQUENCE

As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide". In some instances, the term  
15 "amino acid sequence" is synonymous with the term "protein".

The amino acid sequence may be prepared isolated from a suitable source, or it may be made synthetically or it may be prepared by use of recombinant DNA techniques.

20

#### NUCLEOTIDE SEQUENCE

As used herein, the term "nucleotide sequence" is synonymous with the term "polynucleotide".

25

The nucleotide sequence may be DNA or RNA of genomic or synthetic or of recombinant origin. The nucleotide sequence may be double-stranded or single-stranded whether representing the sense or antisense strand or combinations thereof.

30

For some applications, preferably, the nucleotide sequence is DNA.

For some applications, preferably, the nucleotide sequence is prepared by use of recombinant DNA techniques (e.g. recombinant DNA).

35

For some applications, preferably, the nucleotide sequence is cDNA.



For some applications, preferably, the nucleotide sequence may be the same as the naturally occurring form for this aspect.

5 It will be understood by a skilled person that numerous different nucleotide sequences can encode the targets as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not substantially affect the activity encoded by the nucleotide sequence of the present invention to reflect the codon usage of any particular host organism in which the target is to be expressed. Thus, the terms  
10 "variant", "homologue" or "derivative" in relation to the nucleotide sequence set out in the attached sequence listings include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence encodes a functional target according the present invention (or even an agent according to the present invention if  
15 said agent comprises a nucleotide sequence or an amino acid sequence).

As indicated above, with respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequences shown in the sequence listing herein. More preferably there is at least 95%,  
20 more preferably at least 98%, homology. Nucleotide homology comparisons may be conducted as described above. A preferred sequence comparison program is the GCG Wisconsin Bestfit program described above. The default scoring matrix has a match value of 10 for each identical nucleotide and -9 for each mismatch. The default gap creation penalty is -50 and the default gap extension penalty is -3 for each nucleotide.

25 The present invention also encompasses nucleotide sequences that are capable of hybridising selectively to the sequences presented herein, or any variant, fragment or derivative thereof, or to the complement of any of the above. Nucleotide sequences are preferably at least 15 nucleotides in length, more preferably at least 20, 30, 40 or 50  
30 nucleotides in length. These sequences could be used a probes, such as in a diagnostic kit.

#### VARIANTS/HOMOLOGUES/DERIVATIVES

35 In addition to the specific amino acid sequences and nucleotide sequences mentioned herein, the present invention also encompasses the use of variants,

homologue and derivatives thereof. Here, the term "homology" can be equated with "identity".

5 In the present context, an homologous sequence is taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical. In particular, homology should typically be considered with respect to those regions of the sequence known to be essential for an activity. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it  
10 is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

15 % homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are  
20 performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially  
25 resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

30 However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine  
35 gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce

optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, *Nucleic Acids Research* 12:387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.*, 1990, *J. Mol. Biol.*, 403-410) and the GENWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see *FEMS Microbiol Lett* 1999 174(2): 247-50; *FEMS Microbiol Lett* 1999 177(1): 187-8 and [tatiana@ncbi.nlm.nih.gov](mailto:tatiana@ncbi.nlm.nih.gov)).

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of

similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

Replacements may also be made by unnatural amino acids include; alpha\* and alpha-disubstituted\* amino acids, N-alkyl amino acids\*, lactic acid\*, halide derivatives of natural amino acids such as trifluorotyrosine\*, p-Cl-phenylalanine\*, p-Br-phenylalanine\*, p-I-phenylalanine\*, L-allyl-glycine\*,  $\beta$ -alanine\*, L- $\alpha$ -amino butyric acid\*, L- $\gamma$ -amino butyric acid\*, L- $\alpha$ -amino isobutyric acid\*, L- $\epsilon$ -amino caproic acid\*, 7-amino heptanoic acid\*, L-methionine sulfone\*, L-norleucine\*, L-norvaline\*, p-nitro-L-phenylalanine\*, L-hydroxyproline\*, L-thioprolin\*, methyl derivatives of phenylalanine (Phe) such as 4-methyl-Phe\*, pentamethyl-Phe\*, L-Phe (4-amino)\*, L-Tyr (methyl)\*,

L-Phe (4-isopropyl)\*, L-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxyl acid)\*, L-diaminopropionic acid # and L-Phe (4-benzyl)\*. The notation \* has been utilised for the purpose of the discussion above (relating to homologous or non-homologous substitution), to indicate the hydrophobic nature of the derivative whereas # has been  
5 utilised to indicate the hydrophilic nature of the derivative, #\* indicates amphipathic characteristics.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups  
10 such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or  $\beta$ -alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the  $\alpha$ -carbon substituent group is on the  
15 residue's nitrogen atom rather than the  $\alpha$ -carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., PNAS (1992) 89(20), 9367-9371 and Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134.

### HYBRIDISATION

20

The present invention also encompasses the use of sequences that can hybridise to the target sequences presented herein – such as if the agent is an anti-sense sequence.

25 The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies.

30 Nucleotide sequences of the invention capable of selectively hybridising to the nucleotide sequences presented herein, or to their complement, will be generally at least 75%, preferably at least 85 or 90% and more preferably at least 95% or 98% homologous to the corresponding complementary nucleotide sequences presented herein over a region of at least 20, preferably at least 25 or 30, for instance at least 40,  
35 60 or 100 or more contiguous nucleotides. Preferred nucleotide sequences of the invention will comprise regions homologous to the nucleotide sequence set out in SEQ

ID No 2 of the sequence listings of the present invention preferably at least 80 or 90% and more preferably at least 95% homologous to the nucleotide sequence set out in SEQ ID No 2 of the sequence listings of the present invention.

- 5 The term "selectively hybridizable" means that the nucleotide sequence, when used as a probe, is used under conditions where a target nucleotide sequence is found to hybridize to the probe at a level significantly above background. The background hybridization may occur because of other nucleotide sequences present, for example, in the cDNA or genomic DNA library being screened. In this event, background implies a
- 10 level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10 fold, preferably less than 100 fold as intense as the specific interaction observed with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with  $^{32}\text{P}$ .
- 15 Hybridization conditions are based on the melting temperature ( $T_m$ ) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.
- 20 Maximum stringency typically occurs at about  $T_m - 5^\circ\text{C}$  ( $5^\circ\text{C}$  below the  $T_m$  of the probe); high stringency at about  $5^\circ\text{C}$  to  $10^\circ\text{C}$  below  $T_m$ ; intermediate stringency at about  $10^\circ\text{C}$  to  $20^\circ\text{C}$  below  $T_m$ ; and low stringency at about  $20^\circ\text{C}$  to  $25^\circ\text{C}$  below  $T_m$ . As will be understood by those of skill in the art, a maximum stringency hybridization can be used to identify or detect identical nucleotide sequences while an
- 25 intermediate (or low) stringency hybridization can be used to identify or detect similar or related polynucleotide sequences.

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention under stringent conditions

30 (e.g.  $65^\circ\text{C}$  and  $0.1\times\text{SSC}$  { $1\times\text{SSC} = 0.15\text{ M NaCl}$ ,  $0.015\text{ M Na}_3\text{ Citrate pH } 7.0$ ). Where the nucleotide sequence of the invention is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the present invention. Where the nucleotide sequence is single-stranded, it is to be understood that the complementary sequence of that nucleotide sequence is also included within the scope

35 of the present invention.

Nucleotide sequences which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of sources. In addition, other  
5 viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other  
10 animal species, and probing such libraries with probes comprising all or part of the nucleotide sequence set out in herein under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the amino acid and/or nucleotide sequences of the present invention.

15 Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can  
20 be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used. The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

25 Alternatively, such nucleotide sequences may be obtained by site directed mutagenesis of characterised sequences, such as the nucleotide sequence set out in SEQ ID No 2 of the sequence listings of the present invention. This may be useful where for example silent codon changes are required to sequences to optimise codon preferences for a  
30 particular host cell in which the nucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the activity of the protein encoded by the nucleotide sequences.

The nucleotide sequences of the present invention may be used to produce a primer,  
35 e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the nucleotide sequences may be cloned into vectors. Such

primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term nucleotide sequence of the invention as used herein.

- 5 The nucleotide sequences such as a DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

- 10 In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

- 15 Longer nucleotide sequences will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction (PCR) under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

- 25 Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence, may be used to clone and express the target sequences. As will be understood by those of skill in the art, for certain expression systems, it may be advantageous to produce the target sequences with non-naturally occurring codons. Codons preferred by a particular prokaryotic or eukaryotic host (Murray E *et al* (1989) Nuc Acids Res 17:477-508) can be selected, for example, to increase the rate of the target expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

35

#### EXPRESSION VECTORS



The nucleotide sequence for use as the target or for expressing the target can be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence in and/or from a compatible host cell. Expression may be controlled using control sequences which include  
5 promoters/enhancers and other expression regulation signals. Prokaryotic promoters and promoters functional in eukaryotic cells may be used. Tissue specific or stimuli specific promoters may be used. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

10 The protein produced by a host recombinant cell by expression of the nucleotide sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences can be designed with signal sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

### 15 FUSION PROTEINS

The target amino acid sequence may be produced as a fusion protein, for example to aid in extraction and purification. Examples of fusion protein partners include  
20 glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and  $\beta$ -galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the target.

25 The fusion protein may comprise an antigen or an antigenic determinant fused to the substance of the present invention. In this embodiment, the fusion protein may be a non-naturally occurring fusion protein comprising a substance which may act as an adjuvant in the sense of providing a generalised stimulation of the immune system.

30 The antigen or antigenic determinant may be attached to either the amino or carboxy terminus of the substance.

In another embodiment of the invention, the amino acid sequence may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of  
35 peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognized by a commercially available antibody.

## ANTIBODIES

In one embodiment of the present invention, the agent may be an antibody. In  
5 addition, or in the alternative, the target may be an antibody. In addition, or in the  
alternative, the means for detecting the target may be an antibody.

Antibodies may be produced by standard techniques, such as by immunisation with  
the substance of the invention or by using a phage display library.

10

For the purposes of this invention, the term "antibody", unless specified to the contrary,  
includes but is not limited to, polyclonal, monoclonal, chimeric, single chain, Fab  
fragments, fragments produced by a Fab expression library, as well as mimetics  
thereof. Such fragments include fragments of whole antibodies which retain their  
15 binding activity for a target substance, Fv, F(ab') and F(ab')<sub>2</sub> fragments; as well as single  
chain antibodies (scFv), fusion proteins and other synthetic proteins which comprise  
the antigen-binding site of the antibody. Furthermore, the antibodies and fragments  
thereof may be humanised antibodies. Neutralizing antibodies, i.e., those which inhibit  
biological activity of the substance polypeptides, are especially preferred for  
20 diagnostics and therapeutics.

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat,  
horse, etc.) is immunised with an immunogenic polypeptide bearing a epitope(s)  
obtainable from an identified agent and/or substance of the present invention.

25 Depending on the host species, various adjuvants may be used to increase  
immunological response. Such adjuvants include, but are not limited to, Freund's,  
mineral gels such as aluminium hydroxide, and surface active substances such as  
lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet  
hemocyanin, and dinitrophenol. BCG (*Bacilli Calmette-Guerin*) and *Corynebacterium*  
30 *parvum* are potentially useful human adjuvants which may be employed if purified the  
substance polypeptide is administered to immunologically compromised individuals  
for the purpose of stimulating systemic defence.

Serum from the immunised animal is collected and treated according to known  
35 procedures. If serum containing polyclonal antibodies to an epitope obtainable from  
an identified agent and/or substance of the present invention contains antibodies to  
other antigens, the polyclonal antibodies can be purified by immunoaffinity

chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides polypeptides of the invention or fragments thereof haptened to another polypeptide for use as immunogens in animals or humans.

5

Monoclonal antibodies directed against epitopes obtainable from an identified agent and/or substance of the present invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by  
10 cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against orbit epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

15 Monoclonal antibodies to the substance and/or identified agent may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to, the hybridoma technique originally described by Koehler and Milstein (1975 *Nature* 256:495-497), the human B-cell hybridoma technique (Kosbor *et al* (1983) *Immunol Today* 4:72;  
20 Cote *et al* (1983) *Proc Natl Acad Sci* 80:2026-2030) and the EBV-hybridoma technique (Cole *et al* (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R Liss Inc, pp 77-96). In addition, techniques developed for the production of "chimeric antibodies", the splicing of mouse antibody genes to human antibody genes to obtain a molecule with appropriate antigen specificity and biological activity can be used  
25 (Morrison *et al* (1984) *Proc Natl Acad Sci* 81:6851-6855; Neuberger *et al* (1984) *Nature* 312:604-608; Takeda *et al* (1985) *Nature* 314:452-454). Alternatively, techniques described for the production of single chain antibodies (US Patent No. 4,946,779) can be adapted to produce the substance specific single chain antibodies.

30 Antibodies, both monoclonal and polyclonal, which are directed against epitopes obtainable from an identified agent and/or substance are particularly useful in diagnosis, and those which are neutralising are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotypic antibodies. Anti-idiotypic antibodies are immunoglobulins which carry an "internal image" of the  
35 substance and/or agent against which protection is desired. Techniques for raising anti-idiotypic antibodies are known in the art. These anti-idiotypic antibodies may also be useful in therapy.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi *et al* (1989, Proc Natl Acad Sci 86: 3833-3837), and Winter G and Milstein C (1991; Nature 349:293-299).

Antibody fragments which contain specific binding sites for the substance may also be generated. For example, such fragments include, but are not limited to, the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse WD *et al* (1989) Science 256:1275-128 1).

## REPORTERS

A wide variety of reporters may be used in the assay methods (as well as screens) of the present invention with preferred reporters providing conveniently detectable signals (eg. by spectroscopy). By way of example, a reporter gene may encode an enzyme which catalyses a reaction which alters light absorption properties.

Examples of reporter molecules include but are not limited to  $\beta$ -galactosidase, invertase, green fluorescent protein, luciferase, chloramphenicol, acetyltransferase,  $\beta$ -glucuronidase, exo-glucanase and glucoamylase. Alternatively, radiolabelled or fluorescent tag-labelled nucleotides can be incorporated into nascent transcripts which are then identified when bound to oligonucleotide probes.

In one preferred embodiment, the production of the reporter molecule is measured by the enzymatic activity of the reporter gene product, such as  $\beta$ -galactosidase.

A variety of protocols for detecting and measuring the expression of the target, such as by using either polyclonal or monoclonal antibodies specific for the protein, are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilising monoclonal antibodies reactive to two non-interfering epitopes on polypeptides is preferred, but a competitive binding assay

may be employed. These and other assays are described, among other places, in Hampton R *et al* (1990, Serological Methods, A Laboratory Manual, APS Press, St Paul MN) and Maddox DE *et al* (1983, J Exp Med 15 8:121 1).

- 5 A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays. Means for producing labelled hybridisation or PCR probes for detecting the target polynucleotide sequences include oligolabelling, nick translation, end-labelling or PCR amplification using a labelled nucleotide. Alternatively, the coding sequence, or any portion of it, 10 may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes *in vitro* by addition of an appropriate RNA polymerase such as T7, T3 or SP6 and labelled nucleotides.
- 15 A number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for these procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. 20 Patents teaching the use of such labels include US-A-3817837; US-A-3850752; US-A-3939350; US-A-3996345; US-A-4277437; US-A-4275149 and US-A-4366241. Also, recombinant immunoglobulins may be produced as shown in US-A-4816567.

- Additional methods to quantify the expression of a particular molecule include 25 radiolabeling (Melby PC *et al* 1993 J Immunol Methods 159:235-44) or biotinylating (Duplaa C *et al* 1993 Anal Biochem 229:36) nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated. Quantification of multiple samples may be speeded up by running the assay in an ELISA format where the oligomer of interest is presented in various 30 dilutions and a spectrophotometric or calorimetric response gives rapid quantification.

- Although the presence/absence of marker gene expression suggests that the gene of interest is also present, its presence and expression should be confirmed. For example, if the nucleotide sequence is inserted within a marker gene sequence, 35 recombinant cells containing the same may be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a target coding sequence under the control of a single promoter. Expression of the marker

gene in response to induction or selection usually indicates expression of the target as well.

Alternatively, host cells which contain the coding sequence for the target and express the target coding regions may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridisation and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid or protein.

### SCREENS

Any one or more of appropriate targets - such as an amino acid sequence and/or nucleotide sequence - may be used for identifying a NEPi in any of a variety of drug screening techniques. The target employed in such a test may be free in solution, affixed to a solid support, borne on a cell surface, or located intracellularly. The target may even be within an animal model, wherein said target may be an exogenous target or an introduced target. The animal model will be a non-human animal model. The abolition of target activity or the formation of binding complexes between the target and the agent being tested may be measured.

Techniques for drug screening may be based on the method described in Geysen, European Patent Application 84/03564, published on September 13, 1984. In summary, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with a suitable target or fragment thereof and washed. Bound entities are then detected - such as by appropriately adapting methods well known in the art. A purified target can also be coated directly onto plates for use in a drug screening techniques. Alternatively, non-neutralising antibodies can be used to capture the peptide and immobilise it on a solid support.

This invention also contemplates the use of competitive drug screening assays in which neutralising antibodies capable of binding a target specifically compete with a test compound for binding to a target.

Another technique for screening provides for high throughput screening (HTS) of agents having suitable binding affinity to the substances and is based upon the method described in detail in WO 84/03564.

- 5 It is expected that the assay methods of the present invention will be suitable for both small and large-scale screening of test compounds as well as in quantitative assays.

In a preferred aspect, the screen of the present invention comprises at least the following steps (which need not be in this same consecutive order): (a) conducting an  
10 *in vitro* screen to determine whether a candidate agent has the relevant activity (such as modulation of NEP, such as NEP from dog kidney); (b) conducting one or more selectivity screens to determine the selectivity of said candidate agent (e.g. to see if said agent is also an ACE inhibitor – such as by using the assay protocol presented herein); and (c) conducting an *in vivo* screen with said candidate agent (e.g. using a  
15 functional animal model). Typically, if said candidate agent passes screen (a) and screen (b) then screen (c) is performed.

### DIAGNOSTICS

- 20 The present invention also provides a diagnostic composition or kit for the detection of a pre-disposition for MED. In this respect, the composition or kit will comprise an entity that is capable of indicating the presence of one or more - or even the absence of one or more - of the targets in a test sample. Preferably, the test sample is obtained from the penis.

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By way of example, the diagnostic composition may comprise any one of the nucleotide sequences mentioned herein or a variant, homologue, fragment or derivative thereof, or a sequence capable of hybridising to all or part of any one of the nucleotide sequence.

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- In order to provide a basis for the diagnosis of disease, normal or standard values from a target should be established. This may be accomplished by combining body fluids or cell extracts taken from normal subjects, either animal or human, with an antibody to a target under conditions suitable for complex formation which are well  
35 known in the art. The amount of standard complex formation may be quantified by comparing it to a dilution series of positive controls where a known amount of antibody is combined with known concentrations of a purified target. Then, standard

values obtained from normal samples may be compared with values obtained from samples from subjects potentially affected by MED. Deviation between standard and subject values establishes the presence of the disease state.

- 5 A target itself, or any part thereof, may provide the basis for a diagnostic and/or a therapeutic compound. For diagnostic purposes, target polynucleotide sequences may be used to detect and quantify gene expression in conditions, disorders or diseases in which MED may be implicated.
- 10 The target encoding polynucleotide sequence may be used for the diagnosis of MED resulting from expression of the target. For example, polynucleotide sequences encoding a target may be used in hybridisation or PCR assays of tissues from biopsies or autopsies or biological fluids, to detect abnormalities in target expression. The form of such qualitative or quantitative methods may include Southern or
- 15 northern analysis, dot blot or other membrane-based technologies; PCR technologies; dip stick, pin or chip technologies; and ELISA or other multiple sample formal technologies. All of these techniques are well known in the art and are in fact the basis of many commercially available diagnostic kits.
- 20 Such assays may be tailored to evaluate the efficacy of a particular therapeutic treatment regime and may be used in animal studies, in clinical trials, or in monitoring the treatment of an individual patient. In order to provide a basis for the diagnosis of disease, a normal or standard profile for target expression should be established. This is accomplished by combining body fluids or cell extracts taken from normal
- 25 subjects, either animal or human, with the target or a portion thereof, under conditions suitable for hybridisation or amplification. Standard hybridisation may be quantified by comparing the values obtained for normal subjects with a dilution series of positive controls run in the same experiment where a known amount of purified target is used. Standard values obtained from normal samples may be compared
- 30 with values obtained from samples from subjects potentially affected by a disorder or disease related to expression of the target coding sequence. Deviation between standard and subject values establishes the presence of the disease state. If disease is established, an existing therapeutic agent is administered, and treatment profile or values may be generated. Finally, the assay may be repeated on a regular
- 35 basis to evaluate whether the values progress toward or return to the normal or standard pattern. Successive treatment profiles may be used to show the efficacy of treatment over a period of several days or several months.



Thus, in one aspect, the present invention relates to the use of a target polypeptide, or variant, homologue, fragment or derivative thereof, to produce anti-target antibodies which can, for example, be used diagnostically to detect and quantify target levels in MED.

The present invention further provides diagnostic assays and kits for the detection of a target in cells and tissues comprising a purified target which may be used as a positive control, and anti-target antibodies. Such antibodies may be used in solution-based, membrane-based, or tissue-based technologies to detect any disease state or condition related to the expression of target protein or expression of deletions or a variant, homologue, fragment or derivative thereof.

#### ASSAY METHODS

The diagnostic compositions and/or methods and/or kits may be used in the following techniques which include but are not limited to; competitive and non-competitive assays, radioimmunoassay, bioluminescence and chemiluminescence assays, fluorometric assays, sandwich assays, immunoradiometric assays, dot blots, enzyme linked assays including ELISA, microtiter plates, antibody coated strips or dipsticks for rapid monitoring of urine or blood, immunohistochemistry and immunocytochemistry.

By way of example, an immunohistochemistry kit may also be used for localization of NEP activity in genital tissue. This immunohistochemistry kit permits localization of NEP in tissue sections and cultured cells using both light and electron microscopy which may be used for both research and clinical purposes. Such information may be useful for diagnostic and possibly therapeutic purposes in the detection and/or prevention and/or treatment of MED. For each kit the range, sensitivity, precision, reliability, specificity and reproducibility of the assay are established. Intraassay and interassay variation is established at 20%, 50% and 80% points on the standard curves of displacement or activity.

#### PROBES

Another aspect of the subject invention is the provision of nucleic acid hybridisation or PCR probes which are capable of detecting (especially those that are capable of selectively selecting) polynucleotide sequences, including genomic sequences,

- encoding a target coding region or closely related molecules, such as alleles. The specificity of the probe, i.e., whether it is derived from a highly conserved, conserved or non-conserved region or domain, and the stringency of the hybridisation or amplification (high, intermediate or low) will determine whether the probe identifies only naturally occurring target coding sequence, or related sequences. Probes for the detection of related nucleic acid sequences are selected from conserved or highly conserved nucleotide regions of target family members and such probes may be used in a pool of degenerate probes. For the detection of identical nucleic acid sequences, or where maximum specificity is desired, nucleic acid probes are selected from the non-conserved nucleotide regions or unique regions of the target polynucleotides. As used herein, the term "non-conserved nucleotide region" refers to a nucleotide region that is unique to a target coding sequence disclosed herein and does not occur in related family members.
- PCR as described in US-A-4683195, US-A-4800195 and US-A-4965188 provides additional uses for oligonucleotides based upon target sequences. Such oligomers are generally chemically synthesized, but they may be generated enzymatically or produced from a recombinant source. Oligomers generally comprise two nucleotide sequences, one with sense orientation (5'→3') and one with antisense (3'←5') employed under optimised conditions for identification of a specific gene or condition. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantification of closely related DNA or RNA sequences.
- The nucleic acid sequence for a target can also be used to generate hybridisation probes as previously described, for mapping the endogenous genomic sequence. The sequence may be mapped to a particular chromosome or to a specific region of the chromosome using well known techniques. These include *in situ* hybridisation to chromosomal spreads (Verma *et al* (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York City), flow-sorted chromosomal preparations, or artificial chromosome constructions such as YACs, bacterial artificial chromosomes (BACs), bacterial PI constructions or single chromosome cDNA libraries.
- In situ* hybridisation of chromosomal preparations and physical mapping techniques such as linkage analysis using established chromosomal markers are invaluable in extending genetic maps. Examples of genetic maps can be found in Science (1995;

270:410f and 1994; 265:1981f). Often the placement of a gene on the chromosome of another mammalian species may reveal associated markers even if the number or arm of a particular human chromosome is not known. New sequences can be assigned to chromosomal arms, or parts thereof, by physical mapping. This provides valuable information to investigators searching for disease genes using positional cloning or other gene discovery techniques. Once a disease or syndrome has been crudely localised by genetic linkage to a particular genomic region any sequences mapping to that area may represent associated or regulatory genes for further investigation. The nucleotide sequence of the subject invention may also be used to detect differences in the chromosomal location due to translocation, inversion, etc. between normal, carrier or affected individuals.

### ORGANISM

The term "organism" in relation to the present invention includes any organism that could comprise the target and/or products obtained therefrom. Examples of organisms may include a fungus, yeast or a plant.

The term "transgenic organism" in relation to the present invention includes any organism that comprises the target and/or products obtained.

### TRANSFORMATION OF HOST CELLS/HOST ORGANISMS

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism.

Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook et al (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press) and Ausubel *et al.*, Current Protocols in Molecular Biology (1995), John Wiley & Sons, Inc.

If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

In another embodiment the transgenic organism can be a yeast. In this regard, yeast have also been widely used as a vehicle for heterologous gene expression. The species *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes in

*Saccharomyces cerevisiae* has been reviewed by Goodey et al (1987, Yeast Biotechnology, D R Berry et al, eds, pp 401-429, Allen and Unwin, London) and by King et al (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).

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For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability.

10 Third, the extensive commercial use and research devoted to the organism has resulted in a wealth of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

A review of the principles of heterologous gene expression in *Saccharomyces*  
15 *cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

Several types of yeast vectors are available, including integrative vectors, which require  
20 recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting the nucleotide sequence of the present invention into a construct designed  
25 for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the nucleotide sequence of the present invention, usually a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends  
30 the expression system.

For the transformation of yeast several transformation protocols have been developed. For example, a transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen et al (1978, Proceedings of the National  
35 Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito, H et al (1983, J Bacteriology 153, 163-168).

The transformed yeast cells are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, eg G418.

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Another host organism is a plant. The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material. Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] 42:205-225) and Christou (*Agro-Food-Industry Hi-Tech* March/April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

15

Thus, the present invention also provides a method of transforming a host cell with a nucleotide sequence that is to be the target or is to express the target. Host cells transformed with the nucleotide sequence may be cultured under conditions suitable for the expression and recovery of the encoded protein from cell culture. The protein produced by a recombinant cell may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing coding sequences can be designed with signal sequences which direct secretion of the coding sequences through a particular prokaryotic or eukaryotic cell membrane. Other recombinant constructions may join the coding sequence to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins (Kroll *DJ et al* (1993) *DNA Cell Biol* 12:441-53).

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## NEPi – ANIMAL TEST METHODS

### Animal models

#### Anaesthetised Rabbit Methodology

Male New Zealand rabbits (~2.5kg) were pre-medicated with a combination of Medetomidine (Domitor®) 0.5ml/kg *i.m.*, and Ketamine (Vetalar®) 0.25ml/kg *i.m.* whilst maintaining oxygen intake via a face mask. The rabbits were tracheotomised using a Portex™ uncuffed endotracheal tube 3 ID., connected to ventilator and maintained at a ventilation rate of 30-40 breaths per minute, with an approximate tidal volume of 18-20 ml, and a maximum airway pressure of 10 cm H<sub>2</sub>O. Anaesthesia was then switched to Isoflurane and ventilation continued with O<sub>2</sub> at 2l/min. The right marginal ear v in was cannulated using a 23G or 24G catheter, and

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Lactated Ringer solution perfused at 0.5ml/min. The rabbit was maintained at 3% Isoflurane during invasive surgery, dropping to 2% for maintenance anaesthesia. The left jugular vein was exposed, isolated and then cannulated with a PVC catheter (17G) for the infusion of drugs and compounds. A ventral midline incision was made into the abdominal cavity. The incision was about 5cm in length just above the pubis. The fat and muscle was *bluntly* dissected away to reveal the hypogastric nerve which runs down the body cavity. It was essential to keep close to the side curve of the pubis wall in order to avoid damaging the femoral vein and artery which lie above the pubis. The sciatic and pelvic nerves lie deeper and were located after further dissection on the dorsal side of the rabbit. Once the sciatic nerve is identified, the pelvic nerve was easily located. The term *pelvic nerve* is loosely applied; anatomy books on the subject fail to identify the nerves in sufficient detail. However, stimulation of the nerve causes an increase in vaginal and clitoral blood flow, and innervation of the pelvic region. The pelvic nerve was freed away from surrounding tissue and a *Harvard* bipolar stimulating electrode was placed around the nerve. The nerve was slightly lifted to give some tension, then the electrode was secured in position. Approximately 1ml of light paraffin oil was placed around the nerve and electrode. This acts as a protective lubricant to the nerve and prevents blood contamination of the electrode. The electrode was connected to a *Grass* S88 Stimulator. The pelvic nerve was stimulated using the following parameters:- 5V, pulse width 0.5ms, duration of stimulus 20 seconds with a frequency of 16Hz. Reproducible responses were obtained when the nerve was stimulated every 15-20 minutes. Several stimulations using the above parameters were performed to establish a mean control response. The compound(s) to be tested were infused, via the jugular vein, using a *Harvard* 22 infusion pump allowing a continuous 15 minute stimulation cycle. The skin and connective tissue around the penis was removed to expose the penis. A catheter set (*Insyte-W*, Becton-Dickinson 20 Gauge 1.1 x 48mm) was inserted through the tunica albica into the left *corpus cavernosal* space and the needle removed, leaving a flexible catheter. This catheter was linked via a pressure transducer (Ohmeda 5299-04) to a Gould system to record intracavernosal pressure. Once an intracavernosal pressure was established, the catheter was sealed in place using *Vetbond* (tissue adhesive, 3M). Heart rate was measured via the pulse oxymeter and *Po-ne-mah* data acquisition software system (Ponemah Physiology Platform, Gould Instrument Systems Inc).

Intracavernosal blood flow was recorded either as numbers directly from the Flowmeter using *Po-ne-mah* data acquisition software (Ponemah Physiology Platform, Gould Instrument Systems Inc), or indirectly from Gould chart recorder



trace. Calibration was set at the beginning of the experiment (0-125ml/min/100g tissue). The NEP (Neutral Endopeptidase EC3.4.24.11) inhibitor was made up in saline + 10% 1M NaOH, the phosphodiesterase type 5 (PDE5) inhibitor was made up in saline + 5% 1M HCl. The inhibitors and vehicle controls were infused at a rate of 0.1ml/second. NEP inhibitors and PDE<sub>cAMP</sub> inhibitors were left for 15 minutes prior to pelvic nerve stimulation.

All data are reported as mean  $\pm$  s.e.m.. Significant changes were identified using Student's t-tests.

#### Anaesthetised Dog Methodology

Male beagles, in the range 12-15 kg body weight, were deprived of food overnight. They were anaesthetised with pentobarbitone (30-45mg/kg *i.v.*), and the anaesthesia maintained by a continual infusion of pentobarbitone (60mg/ml) at a rate of 1-1.4ml/h. The left femoral artery was cannulated for the measurement of blood pressure, lead II E.C.G. was recorded and heart-rate derived. A catheter was introduced into the left femoral vein for the administration of compounds. Both ureters were cannulated *via* a mid-line abdominal incision to prevent urine accumulation in the bladder and the bladder was completely emptied. The left internal pudendal artery was carefully dissected free of surrounding tissues to allow placement of a Transonic flow probe for the measurement of arterial blood flow. The cavernosal branches of both pelvic nerves were dissected free and placed into bipolar stimulating electrodes. The skin around the penis was opened and the corpora cavernosa exposed. A 21g needle, attached by flexible catheter to a pressure transducer, was inserted into the corpus (usually the left) for measurement of both *i.c.* pressure and injection of SNP; the system was filled with heparinised saline (15 to 20 U/ml). In the dog the corpora are totally separate which enabled either or both sides to be used if necessary.

The dogs were respired with a Ugo Basile 5025 dog ventilator adjusted to maintain blood gasses in the range pO<sub>2</sub> 95-115 mmHg; pCO<sub>2</sub> 25-40 mmHg. Expired air was continually monitored by a Datex Normocap 200 to aid respiratory control. Body temperature was maintained within the range 36-38°C using an electric blanket. Parameters were recorded on a Gould TA4000 polygraph and all data acquisition and calculation of derived parameters was carried out on-line using a Po-Ne-Mah system. The cavernosal branches of the pelvic nerves were stimulated with a Grass

S88 stimulator at 10 volts, 2 ms duration for <1 min. At the end of the experiment dogs were killed by an *i.v.* injection of 20ml saturated potassium chloride, whilst still under pentobarbitone anaesthesia. Following a period of equilibration, the pelvic nerves were stimulated at 16Hz in order to assess whether the rise in *i.c.* pressure was rapidly and fully registered by the transducer and changes in blood flow were detected. Control responses were obtained to nerve stimulation at either 1 or 2Hz, On recovery a second stimulation was performed, at double the first frequency. In some dogs a third frequency was used. This stimulation cycle was repeated after 30 min. NEP inhibitors were dissolved in alkaline saline and given as a series of two-tiered infusions starting with a loading infusion and a maintenance infusion for 30 minutes, when the second set of infusions was started. Subsequent infusions were started either at 30 min intervals or when *i.c.* pressure had returned to baseline. All infusions were given at a rate of 1ml/min. Stimulation cycles were started fifteen minutes into each infusion.

15

In addition, arterial blood samples were taken from the abdominal aorta, *via* the blood pressure cannula, pre-dose and at 15 and 30minutes into each infusion, for subsequent analysis of unbound compound concentration by Drug Metabolism.

20

#### NEPi - TEST RESULTS and DISCUSSION

There are a number of anaesthetised animal models of erection which mimic the physiology of penile erection, i.e. increases in penile blood flow and intracavernosal pressure. The effects of sexual arousal are mimicked by stimulation of pelvic neurones that innervate the penis. This is an accepted mechanism to investigate erectile mechanisms and to assess the potential of potential therapeutic agents for the treatment of MED.

It is well established that selective PDE5 inhibitors such as sildenafil enhance nerve stimulated-increases in intracavernosal pressure (ICP) in animal models and that nerve stimulation mimics the erectile process observed in man (Carter et al., 1998, Traish et al., 1999, Omote 1999, Wallis 1999). This PDE5 inhibitor-induced enhancement of ICP characterises the mechanism of action of PDE5 inhibitors and explains how agents such as sildenafil overcomes any relaxant deficiencies associated with MED or impotence. In agreement with these previous studies, the examples hereinafter have demonstrated that a selective PDE5 inhibitor,

administered intravenously, potentiates nerve-stimulated increases in ICP in the anaesthetised rabbit and dog (Examples 2, 4, 5).

5 The examples hereinafter demonstrate that inhibition of NEP EC3.4.24.11 with a selective NEP inhibitor dose-dependently potentiates nerve stimulated increases in intracavernosal pressure in the anaesthetised dog (Examples 1, 2 and 3). At the doses used in this study a similar enhancement of the erectile process was observed with a NEPi as was observed with a PDE5 inhibitor (Example 2). Simultaneously recording intracavernosal pressure (ICP) and cavernosal blood flow illustrated that a  
10 selective NEP inhibitor enhanced both ICP and cavernosal blood flow (Example 3). These examples underline the potential clinical application of a NEP inhibitor therapy to enhance the erectile process and hence in the treatment of MED.

Examples 4 and 5 demonstrate that concomitant inhibition of NEP EC3.4.24.11 and  
15 PDE5 produced a marked enhancement of the ICP, or the erectile process, than was achievable with the same dose of the same PDE5 inhibitor alone. Using the rabbit model of erection, it has been demonstrated that the potentiation of ICP induced by PDE5 inhibition can be further potentiated by co-administration of a NEP EC3.4.24.11 inhibitor (via intravenous administration of a NEPi, 1mg/kg; Example 4).  
20 At 1mg/kg (iv) doses of PDE5 inhibitor we observe a maximal potentiation of ICP, the finding that the ICP can be further potentiated beyond this maximal PDE5 inhibitor mediated is highly unexpected. This data illustrates that there are a number of clinical benefits of concomitant administration of a PDE5 inhibitor and a NEP inhibitor over PDE5 inhibitor therapy alone. These include increased efficacy and  
25 opportunities to treat MED subgroups that do not respond to PDE5 inhibitor therapy.

It is a particular object of the present invention to provide pharmaceutical compositions comprising a NEPi and a PDEi for use in the treatment of MED wherein the specific combination provides synergistic benefits.

30

In addition the onset of action of PDE5 inhibitors i.e. the time taken to reach maximal effect is greatly reduced in the presence of a NEP EC 3.4.24.11 inhibitor (Example 5). Clinically this represents a quicker onset time.

35 In addition, co-administration of a NEPi and a PDE5i allows the onset of action of PDE5i to be reduced. Hence there is a quickening of the time between agent administration and clinical endpoint.

Inhibitors of NEP EC3.4.24.11 and PDE5 or combinations of the two, have no significant effect on un-stimulated ICP i.e. they do not directly induce an increase in ICP in the absence of sexual drive/arousal. This is highly advantageous as the only  
5 other marketed therapy for MED which requires sexual stimulation to work is sildenafil thus the present invention provides a viable alternative oral therapy to sildenafil and all other PDE5 alone based drugs.

## NEPi - ANIMAL MODEL EXAMPLES

Compounds used:

NEPi: a preferred compound of general formula I as defined herein before.

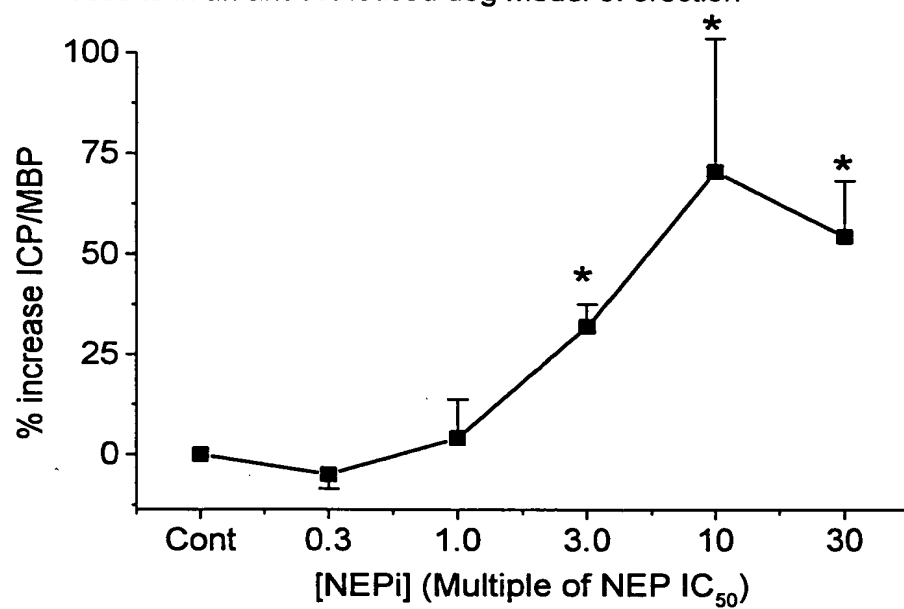
- 5 PDE5i: 3-ethyl-5-{5-[4-ethylpiperzino]-2-propoxyphenyl}-2-(-pyridylmethyl)-6,7-dihydro-2H-pyrazolo[4,3-*d*]pyrimidin-7-one.

- 10 Example 1. Inhibition of NEP EC3.4.24.11 dose-dependently potentiates nerve stimulated increases in intracavernosal pressure in anaesthetised dog model of erection.

- 15 Submaximal increases in intracavernosal pressure (ICP) induced by nerve-stimulation were significantly increased in the presence of increasing doses of a selective NEP EC3.4.24.11 inhibitor (iv infusion to steady state concentrations). The maximal potentiation (circa 70%) was observed at around 10 times the IC50 value obtained against native NEP. Data is expressed as the percentage (%) increase, compared to control stimulated increases, in ICP divided by mean blood pressure (MBP) and multiplied by 100. Values are expressed as mean  $\pm$  s.e.mean. \* P<0.01,  
20 Students t-test unpaired compared with control increases.

There were no major effects of NEP inhibition on basal/un-stimulated intracavernosal pressure.

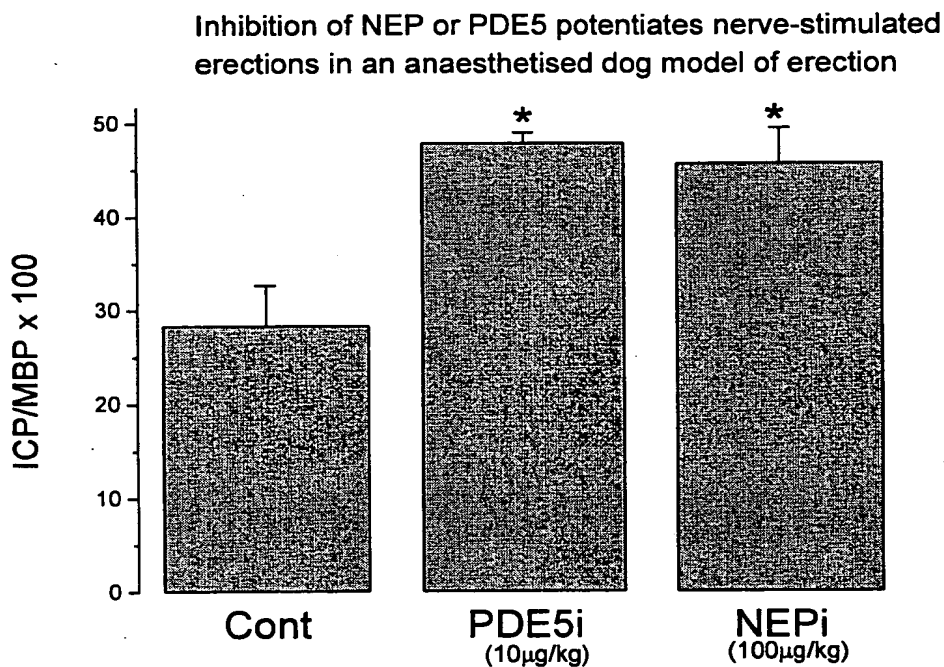
Inhibition of NEP dose-dependantly potentiates nerve-stimulated erections in an anaesthetised dog model of erection



**Example 2. Inhibition of PDE5 or NEP EC3.4.24.11 potentiates nerve stimulated increases in intracavernosal pressure in anaesthetised dog model of erection.**

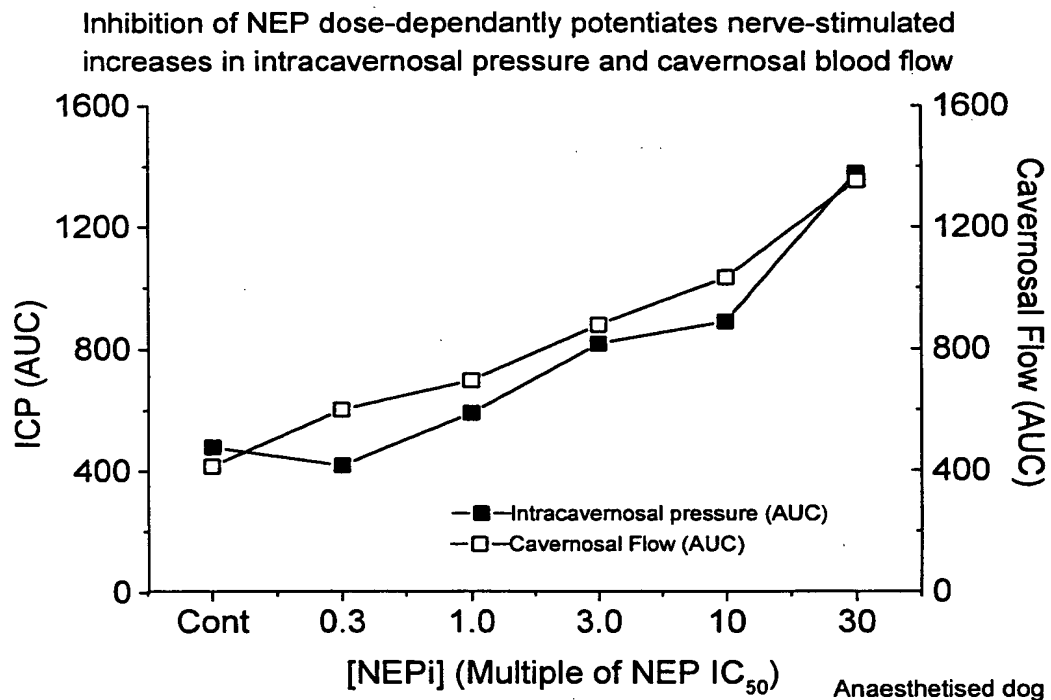
Submaximal increases in intracavernosal pressure (ICP) induced by nerve-stimulation were significantly increased in the presence of a selective PDE5 inhibitor (10µg/kg; iv bolus) NEP EC3.4.24.11 inhibitor (100µg/kg; iv bolus). The maximal potentiation for both agents was circa 65% at the doses used. Data is expressed as ICP divided by mean blood pressure (MBP) and multiplied by 100. Values are expressed as mean  $\pm$  s.e.mean. \* P<0.01, Students t-test unpaired compared with control increases.

There were no major effects of NEP or PDE5 inhibition on basal/un-stimulated intracavernosal pressure.



Example 3. NEP inhibition dose-dependently potentiates nerve stimulated increases in intracavernosal pressure and cavernosal blood flow in anaesthetised dog model of erection.

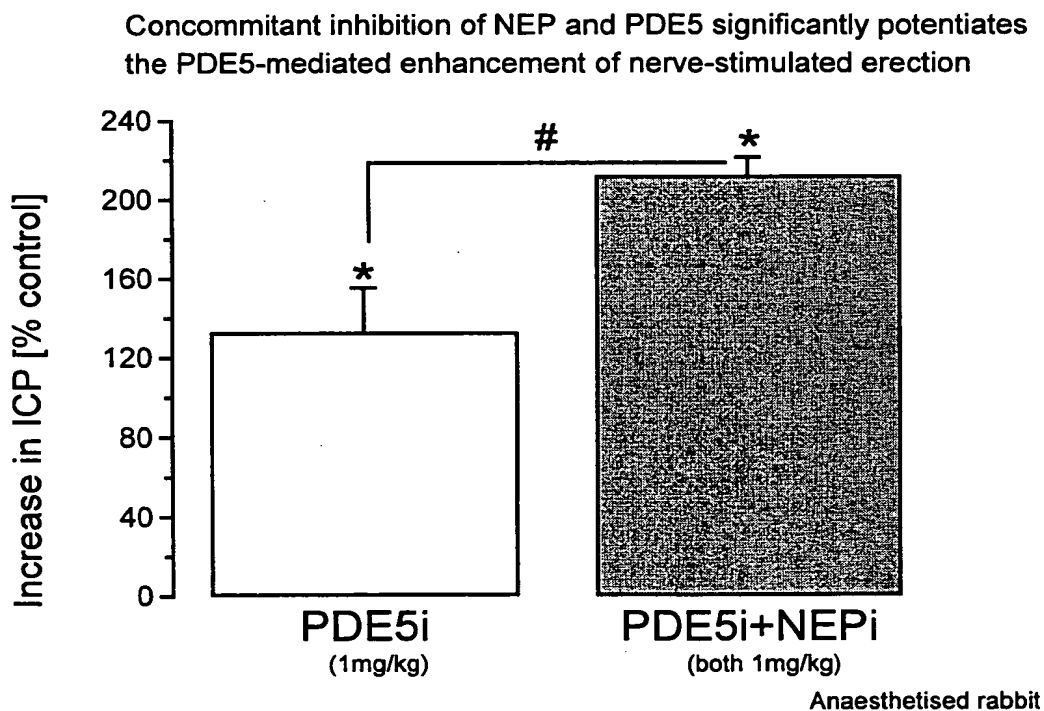
- 5 Submaximal increases in intracavernosal pressure (ICP) and increases in cavernosal blood flow induced by nerve-stimulation were increased in the presence of increasing doses of a selective NEP EC3.4.24.11 inhibitor (iv infusion to steady state concentrations). ICP was increased circa 188% whereas flow was increased circa 228%. Data for ICP and flow, both expressed as area under the curve (AUC), were
- 10 recorded simultaneously from a single animal.





Example 4. NEP inhibition significantly increases the efficacy of PDE5 inhibitor to enhance penile erection in an anaesthetised rabbit model of erection.

Intravenous administration of a selective PDE5 inhibitor (1 mg/kg) significantly enhanced nerve-stimulated increases in ICP by  $133 \pm 22\%$  compared to control increases. Once the PDE5i-mediated increase was sustained, co-administration of a selective NEP EC3.4.24.11 inhibitor further enhanced nerve-stimulated increases in ICP. This represents a NEP inhibition-induced potentiation of 79% ( $P < 0.01$ , paired t-test) compared to increases observed with a PDE5 inhibitor. Data is expressed as percentage increase in ICP over control increases. Values are expressed as mean  $\pm$  s.e.mean. \*  $P < 0.01$ , Students t-test unpaired compared with control increases. There were no effects of PDE5 inhibition or combined PDE5/NEP inhibition on basal/un-stimulated intracavernosal pressure.



Example 5. NEP inhibition potentiates the erectile effects of PDE5 inhibitors and speeds up the onset of action of PDE5 inhibitors in the anaesthetised rabbit model of erection.

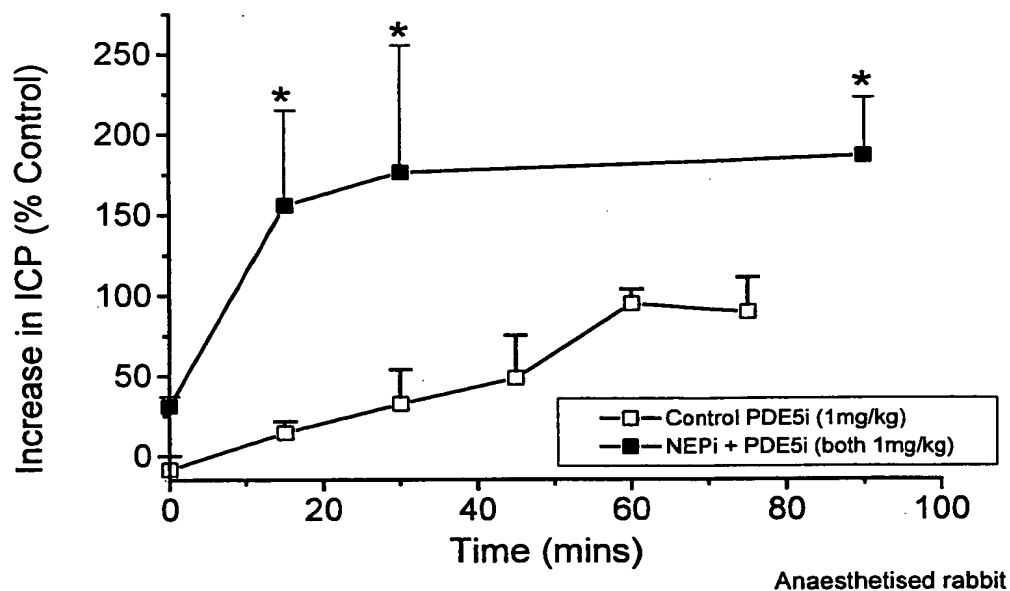
- 5 Concomitant inhibition of NEP EC3.4.24.11 and PDE5 significantly potentiates the PDE5 inhibitor-mediated enhancement of nerve-stimulated increases in intracavernosal pressure (ICP). Submaximal increases in ICP are significantly enhanced (circa 90% compared to control increases) in the presence of a selective PDE5 inhibitor (1mg/kg; iv bolus). When the same dose of the PDE5 inhibitor is given  
10 in the presence of a NEP inhibitor (1mg/kg; iv bolus) a further enhancement of ICP is observed (circa 187% compared to control increases). This represents a NEP inhibitor mediated enhance of PDE5 inhibitor mediated effects of around 100%.

- 15 In addition to the increased enhancement of ICP observed on concomitant application of a NEPi and a PDE5i, the time taken for a PDE5 inhibitor to exert it's maximal effect (i.e. onset of action) is reduced in the presence of a NEP inhibitor (22.5 min in the presence compared to 67.5 min in the absence of a NEP inhibitor).

- 20 There were no major effects of NEP inhibition or combined PDE5/NEP inhibition on basal/un-stimulated intracavernosal pressure.

Data is expressed as percentage increase in ICP over control increases. Values are expressed as mean  $\pm$  s.e.mean. \*  $P < 0.01$ , Students t-test unpaired compared with PDE5 inhibitor mediated increases.

Concomitant inhibition of NEP and PDE5 significantly potentiates the PDE5i-mediated enhancement of nerve-stimulated erection



### NEP ENZYME ASSAY

- 5 THE PREPARATION AND ASSAY OF SOLUBLE (NEP) NEUTRAL ENDOPEPTIDASE FROM CANINE, RAT, RABBIT AND HUMAN KIDNEY CORTEX.

Soluble NEP is obtained from the kidney cortex and activity is assayed by measuring the rate of cleavage of the NEP substrate Abz-D-Arg-Arg-Leu-EDDnp to generate its  
10 fluorescent product, Abz-D-Arg-Arg.

### EXPERIMENTAL PROCEDURE:-

#### 15 1. MATERIALS

All water is double de ionised.

##### 1.1 Tissues

Human Kidney

IIAM (Pennsylvania. U.S.A.)

20 Rat Kidney

Rabbit Kidney

## Canine Kidney

### 1.2 Homogenisation medium

100mM Mannitol and 20mM Tris @ pH 7.1

- 5 2.42g Tris (Fisher T/P630/60) is diluted in 1 litre of water and the pH adjusted to 7.1 using 6M HCl at room temperature. To this 18.22g Mannitol (Sigma M-9546) is added.

### 1.3 Tris buffer (NEP buffer).

- 10 50ml of 50mM Tris pH 7.4 (Sigma T2663) is diluted in 950ml of water.

### 1.4 Substrate (Abz-D-Arg-Arg-Leu-EDDnp)

- Made to order from SNPE, and is stored as a powder at  $-20^{\circ}\text{C}$ . A 2mM stock is made by gently re-suspending the substrate in Tris buffer, this should not be vortexed or  
15 sonicated. 600 $\mu\text{l}$  aliquots of the 2mM stock are stored at  $-20$  for up to one month. (Medeiros, M.A.S., Franca, M.S.F. et al., (1997), Brazilian Journal of Medical and Biological Research, 30, 1157-1162).

### 1.5 Total product

- 20 Samples corresponding to 100% substrate to product conversion are included on the plate to enable the % substrate turnover to be determined. The total product is generated by incubating 1ml of 2mM substrate with 20 $\mu\text{l}$  of enzyme stock for 24 hours at  $37^{\circ}\text{C}$ .

- 25 1.6 Stop solution.

A 300 $\mu\text{M}$  stock of Phosphoramidon (Sigma R7385) is made up in NEP buffer and stored in 50 $\mu\text{l}$  aliquots at  $-20$ .

### 1.7 Dimethyl sulphoxide (DMSO).

- 30 1.8 Magnesium Chloride  $\text{-MgCl}_2 \cdot 6\text{H}_2\text{O}$  (Fisher M0600/53).

### 1.9 Black 96 well flat bottom assay plates (Costar 3915).

### 1.10 Topseal A (Packard 6005185).

### 1.11 Centrifuge tubes

## 35 2. SPECIFIC EQUIPMENT

### 2.1 Sorvall RC-5B centrifuge (SS34 GSA rotor, pre-cooled to $4^{\circ}\text{C}$ ).

### 2.2 Braun miniprimer mixer.

- 2.3 Beckman CS-6R centrifuge.
- 2.4 Fluostar galaxy.
- 2.5 Wesbart 1589 shaking incubator.

## 5 3. METHODS

### 3.1 TISSUE PREPARATION

3.2 Dog, rat, rabbit, and human NEP is obtained from the kidney cortex using a method adapted from Booth, A.G. & Kenny, A.J. (1974) *Biochem. J.* 142, 575-581.

3.3 Frozen kidneys are allowed to thaw at room temperature and the cortex is  
10 dissected away from the medulla.

3.4 The cortex is finely chopped and homogenised in approximately 10 volumes of homogenisation buffer (1.2) using a Braun miniprimer (2.2).

3.5 Magnesium chloride (1.8) (20.3mg/gm tissue) is added to the homogenate and stirred in an ice-water bath for 15 minutes.

15 3.6 The homogenate is centrifuged at 1,500g (3,820rpm) for 12 minutes in a Beckman centrifuge (2.3) before removing the supernatant to a fresh centrifuge tube and discarding the pellet.

3.7 The supernatant is centrifuged at 15,000g (12,100rpm) for 12 minutes in a Sovall centrifuge (2.1) and the supernatant is discarded.

20 3.8 The pale pink layer on the top of the remaining pellet is removed and re-suspended in homogenisation buffer containing magnesium chloride (9mg MgCl in 5ml buffer per 1g tissue).

3.9 The suspension is centrifuged at 2,200g (4,630rpm) for 12 minutes in a Beckman centrifuge (2.3) before discarding the pellet.

25 3.10 The supernatant is centrifuged at 15,000g (12,100rpm) for 12 minutes using the Sorvall centrifuge (2.1) and the supernatant is discarded.

3.11 The final pellet is resuspended in homogenisation buffer containing magnesium chloride (0.9mg MgCl in 0.5ml buffer per 1g tissue). A homogenous suspension is obtained using a Braun miniprimer (2.2). This is then frozen down in

30 100µl aliquots to be assayed for NEP activity.

## 4.0 DETERMINATION OF NEP ACTIVITY

The activity of the previously aliquoted NEP is measured by its ability to cleave the  
35 NEP specific peptide substrate.

4.1 A 4% DMSO/NEP buffer solution is made (4mls DMSO in 96mls NEP buffer).

4.2 Substrate, total product, enzyme, and Phosphoramidon stocks are left on ice to thaw.

4.3 50µl of 4% DMSO/NEP buffer solution is added to each well.

4.4 The 2mM substrate stock is diluted 1:40 to make a 50µM solution. 100µl of  
5 50µM substrate is added to each well (final concentration 25µM).

4.5 50µl of a range of enzyme dilutions is added to initiate the reaction (usually 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200 are used). 50µl of NEP buffer is added to blank wells.

4.6 The 2mM total product is diluted 1:80 to make a 25µM solution. 200µl of  
10 25µM product is added to the first four wells of a new plate.

4.7 Plates are incubated at 37°C in a shaking incubator for 60 minutes.

4.8 The 300µM Phosphoramidon stock is diluted 1:100 to 300nM. The reaction is stopped by the addition of 100µl 300nM Phosphoramidon and incubated at 37°C in a shaking incubator for 20 minutes before being read on the Fluostar (ex320/em420).

## 5. NEP INHIBITION ASSAYS

5.1 Substrate, total product, enzyme and Phosphoramidon stocks are left on ice to thaw.

5.2 Compound stocks are made up in 100% DMSO and diluted 1:25 in NEP buffer to give a 4% DMSO solution. All further dilutions are carried out in a 4% DMSO solution (4mls DMSO in 96mls NEP buffer).

5.3 50µl of compound in duplicate is added to the 96 well plate and 50µl of 4% DMSO/NEP buffer is added to control and blank wells.

5.4 The 2mM substrate stock is diluted 1:40 in NEP buffer to make a 50µM solution (275µl 2mM substrate to 10.73ml buffer is enough for 1 plate).

5.5 The enzyme stock diluted in NEP buffer (determined from activity checks).

5.6 The 2mM total product stock is diluted 1:80 in NEP buffer to make a 25µM solution. 200µl is added to the first four wells of a separate plate.

5.7 The 300µM Phosphoramidon stock is diluted 1:1000 to make a 300nM stock (11µl Phosphoramidon to 10.99ml NEP buffer).

5.8 To each well in the 96 well plate the following is added:

Table Reagents to be added to 96 well plate.

	Compound/ DMSO	Tris Buffer	Substrate	NEP enzyme	Total product
Samples	2µl compound	50µl	100µl	50µl	None
Controls	2µl DMSO	50µl	100µl	50µl	None
Blanks	2µl DMSO	100µl	100µl	None	None
Totals	2µl DMSO	None	None	None	200µl

5.9 The reaction is initiated by the addition of the NEP enzyme before incubating at 37°C for 1 hour in a shaking incubator.

5.10 The reaction is stopped with 100µl 300nM Phosphoramidon and incubated at 37°C for 20 minutes in a shaking incubator before being read on the Fluostar (ex320/em420).

## 6. CALCULATIONS

The activity of the NEP enzyme is determined in the presence and absence of  
5 compound and expressed as a percentage.

% Control activity (turnover of enzyme):

$$10 \quad \frac{\text{Mean FU of controls} - \text{Mean FU of blanks}}{\text{Mean FU of totals} - \text{Mean FU of blanks}} \times 100$$

% Activity with inhibitor:

$$15 \quad \frac{\text{Mean FU of compound} - \text{Mean FU of blanks}}{\text{Mean FU of totals} - \text{Mean FU of blanks}} \times 100$$

Activity expressed as % of control:

$$20 \quad \frac{\% \text{ Activity with inhibitor}}{\% \text{ Control activity}} \times 100$$

A sigmoidal dose-response curve is fitted to the % activities (% of control) vs  
25 compound concentration and IC50 values calculated using LabStats fit-curve in Excel.

### ACE ASSAY

30 THE PREPARATION AND ASSAY OF SOLUBLE ANGIOTENSIN CONVERTING ENZYME (ACE), FROM PORCINE AND HUMAN KIDNEY CORTEX.

Soluble ACE activity is obtained from the kidney cortex and assayed by measuring  
the rate of cleavage of the ACE substrate Abz-Gly-p-nitro-Phe-Pro-OH to generate  
its fluorescent product, Abz-Gly.

#### 35 1. MATERIALS

All water is double de ionised.

##### 1.1 Human Kidney

IIAM (Pennsylvania. U.S.A.) or UK Human



## Tissue Bank (UK HTB)

1.2 Porcine kidney ACE Sigma (A2580)

1.3 Homogenisation buffer-1

100mM Mannitol and 20mM Tris @ pH 7.1

5 2.42g Tris (Fisher T/P630/60) is diluted in 1 litre of water and the pH adjusted to 7.1 using 6M HCl at room temperature. To this 18.22g Mannitol (Sigma M-9546) is added.

1.4 Homogenisation buffer-2

100mM Mannitol, 20mM Tris @ pH7.1 and 10mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (Fisher M0600/53)

10 To 500ml of the homogenisation buffer 1 (1.4) 1.017g of  $\text{MgCl}_2$  is added.

1.5 Tris buffer (ACE buffer).

50mM Tris and 300mM NaCl @ pH 7.4

50ml of 50mM Tris pH 7.4 (Sigma T2663) and 17.52g NaCl (Fisher S/3160/60) are made up to 1000ml in water.

15 1.6 Substrate (Abz-D-Gly-p-nitro-Phe-Pro-OH) (Bachem M-1100)

ACE substrate is stored as a powder at  $-20^\circ\text{C}$ . A 2mM stock is made by gently re-suspending the substrate in ACE buffer, this must not be vortexed or sonicated. 400 $\mu\text{l}$  aliquots of the 2mM stock are stored at  $-20^\circ\text{C}$  for up to one month.

1.7 Total product

20 Samples corresponding to 100% substrate to product conversion are included on the plate to enable the % substrate turnover to be determined (see calculations). The total product is generated by incubating 1ml of 2mM substrate with 20 $\mu\text{l}$  of enzyme stock for 24 hours at  $37^\circ\text{C}$ .

1.8 Stop solution.

25 0.5M EDTA (Promega CAS[6081/92/6]) is diluted 1:250 in ACE buffer to make a 2mM solution.

1.9 Dimethyl sulphoxide (DMSO).

1.10 Magnesium Chloride  $-\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (Fisher M0600/53).

1.11 Black 96 well flat bottom assay plates (Costar 3915 or Packard).

30 1.12 Topseal A (Packard 6005185).

1.13 Centrifuge tubes

## 2. SPECIFIC EQUIPMENT

35 2.1 Sorvall RC-5B centrifuge (SS34 GSA rotor, pre-cooled to  $4^\circ\text{C}$ ).

2.2 Braun miniprimer mixer.

2.3 Beckman CS-6R centrifuge.

- 2.4 BMG Fluostar Galaxy.
- 2.5 Wesbart 1589 shaking incubator.

### 3. METHODS

5

#### 3.1 TISSUE PREPARATION

3.3 Human ACE is obtained from the kidney cortex using a method adapted from Booth, A.G. & Kenny, A.J. (1974) *Biochem. J.* 142, 575-581.

10

3.3 Frozen kidneys are allowed to thaw at room temperature and the cortex is dissected away from the medulla.

3.4 The cortex is finely chopped and homogenised in approximately 10 volumes of homogenisation buffer-1 (1.4) using a Braun miniprimer (2.2).

3.5 Magnesium chloride (1.11) (20.3mg/gm tissue) is added to the homogenate and stirred in an ice-water bath for 15 minutes.

15

3.6 The homogenate is centrifuged at 1,500g (3,820rpm) for 12 minutes in a Beckman centrifuge (2.3) before removing the supernatant to a fresh centrifuge tube and discarding the pellet.

3.7 The supernatant is centrifuged at 15,000g (12,100rpm) for 12 minutes in a Sovall centrifuge (2.1) and the supernatant is discarded.

20

3.8 The pale pink layer on the top of the remaining pellet is removed and re-suspended in homogenisation buffer-2 (1.5) (5ml buffer per 1g tissue).

3.9 The suspension is centrifuged at 2,200g (4,630rpm) for 12 minutes in a Beckman centrifuge before discarding the pellet.

25

3.10 The supernatant is centrifuged at 15,000g (12,100rpm) for 12 minutes using the Sorvall centrifuge and the supernatant is discarded.

3.11 The final pellet is resuspended in homogenisation buffer-2 (0.5ml buffer per 1g tissue). A homogenous suspension is obtained using a Braun miniprimer. This is then frozen down in 100µl aliquots to be assayed for NEP activity.

30

#### 4.0 DETERMINATION OF ACE ACTIVITY

The activity of the previously aliquoted ACE is measured by its ability to cleave the ACE specific peptide substrate.

35

Porcine ACE (1.2) is defrosted and resuspended in ACE buffer (1.6) at 0.004U/µl, this is frozen down in 50µl aliquots.

4.1 A 4% DMSO/ACE buffer solution is made (4mls DMSO in 96mls ACE buffer).

4.2 Substrate (1.7), total product (1.8) and enzyme (1.1, 1.2, 1.3), are left on ice to thaw.

4.3 50µl of 4% DMSO/ACE buffer solution is added to each well.

4.4 The 2mM substrate stock is diluted 1:100 to make a 20µM solution. 100µl of  
5 20µM substrate is added to each well (final concentration in the assay 10µM).

4.5 50µl of a range of enzyme dilutions is added to initiate the reaction (usually 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200 are used). 50µl of ACE buffer is added to blank wells.

4.6 The 2mM total product is diluted 1:200 to make 10µM solution. 200µl 10µM  
10 product is added to the first four wells of a new plate.

4.7 Plates are incubated at 37°C in a shaking incubator for 60 minutes.

4.8 The enzyme reaction is stopped by the addition of 100µl 2mM EDTA in ACE buffer and incubated at 37°C in a shaking incubator for 20 minutes before being read on the BMG Fluostar Galaxy (ex320/em420).

15

## 5. ACE INHIBITION ASSAYS

5.1 Substrate, total product, and enzyme stocks are left on ice to thaw.

5.2 Compound stocks are made up in 100% DMSO and diluted 1:25 in ACE  
20 buffer to give a 4% DMSO solution. All further dilutions are carried out in a 4% DMSO/ACE buffer solution (4mls DMSO in 96mls ACE buffer).

5.3 50µl of compound, in duplicate, is added to the 96 well plate and 50µl of 4% DMSO/ACE buffer is added to control and blank wells.

5.4 Steps 5.2 and 5.3 can be carried out either by hand or using the Packard  
25 multiprobe robots

5.5 The 2mM substrate stock is diluted 1:100 in ACE buffer to make a 20µM solution (10µM final concentration in the assay) (110µl of 2mM substrate added to 10.89ml buffer is enough for 1 plate).

5.6 The enzyme stock is diluted in ACE buffer, as determined from activity checks  
30 (4.0).

5.7 The 2mM total product stock is diluted 1:200 in ACE buffer to make a 10µM solution. 200µl is added to the first four wells of a separate plate.

5.8 The 0.5mM EDTA stock is diluted 1:250 to make a 2mM stock (44µl EDTA to 10.96ml ACE buffer).

35 5.9 To each well of the 96 well plate the following reagents are added:

Table 1: Reagents added to 96 well plate.

	Compound/ DMSO	Tris Buffer	Substrate	ACE enzyme	Total product
Samples	2µl compound	50µl	100µl	50µl	None
Controls	2µl DMSO	50µl	100µl	50µl	None
Blanks	2µl DMSO	100µl	100µl	None	None
Totals	2µl DMSO	None	None	None	200µl

5.10 50µl of the highest concentration of each compound used in the assay is added in duplicate to the same 96 well plate as the totals (5.7). 150µl of ACE buffer is added to determine any compound fluorescence.

5.11 The reaction is initiated by the addition of the ACE enzyme before incubating at 37°C for 1 hour in a shaking incubator.

5.12 The reaction is stopped by the addition of 100µl 2mM EDTA and incubated at 37°C for 20 minutes in a shaking incubator, before being read on the BMG Fluostar Galaxy (ex320/em420).

## 6. CALCULATIONS

The activity of the ACE enzyme is determined in the presence and absence of compound and expressed as a percentage.

FU = Fluorescence units

(i) % Control activity (turnover of enzyme):

10

$$\frac{\text{Mean FU of controls} - \text{Mean FU of blanks}}{\text{Mean FU of totals} - \text{Mean FU of blanks}} \times 100$$

(ii) % Activity with inhibitor:

15

$$\frac{\text{Mean FU of compound} - \text{Mean FU of blanks}}{\text{Mean FU of totals} - \text{Mean FU of blanks}} \times 100$$

(iii) Activity expressed as % of control:

20

$$\frac{\text{\% Activity with inhibitor}}{\text{\% Control activity}} \times 100$$

$$\text{OR} \quad \frac{\text{Mean FU of compound} - \text{Mean FU of blanks}}{\text{Mean FU of controls} - \text{Mean FU of blanks}} \times 100$$

25

(iv) % Inhibition = 100 - % control

(v) For fluorescent compounds the mean FU of blanks containing compound (5.10) is deducted from the mean FU of compound values used to calculate the % Activity.

30

A sigmoidal dose-response curve is fitted to the % activities (% of control) vs compound concentration and IC<sub>50</sub> values calculated using LabStats fit-curve in Excel.

35

## PDE5 inhibitor – TEST METHODS

### Phosphodiesterase (PDE) inhibitory activity

Preferred PDE compounds suitable for use in accordance with the present invention are potent and selective cGMP PDE5 inhibitors. *In vitro* PDE inhibitory activities against cyclic guanosine 3',5'-monophosphate (cGMP) and cyclic adenosine 3',5'-monophosphate (cAMP) phosphodiesterases can be determined by measurement of their  $IC_{50}$  values (the concentration of compound required for 50% inhibition of enzyme activity).

- 10 The required PDE enzymes can be isolated from a variety of sources, including human corpus cavernosum, human and rabbit platelets, human cardiac ventricle, human skeletal muscle and bovine retina, essentially by the method of W.J. Thompson and M.M. Appleman (Biochem., 1971, 10, 311). In particular, the cGMP-specific PDE (PDE5) and the cGMP-inhibited cAMP PDE (PDE3) can be obtained
- 15 from human corpus cavernosum tissue, human platelets or rabbit platelets; the cGMP-stimulated PDE (PDE2) was obtained from human corpus cavernosum; the calcium/calmodulin (Ca/CAM)-dependent PDE (PDE1) from human cardiac ventricle; the cAMP-specific PDE (PDE4) from human skeletal muscle; and the photoreceptor PDE (PDE6) from bovine retina. Phosphodiesterases 7-11 can be generated from full
- 20 length human recombinant clones transfected into SF9 cells.

- Assays can be performed either using a modification of the "batch" method of W.J. Thompson et al. (Biochem., 1979, 18, 5228) or using a scintillation proximity assay for the direct detection of AMP/GMP using a modification of the protocol described by
- 25 Amersham plc under product code TRKQ7090/7100. In summary, the effect of PDE inhibitors was investigated by assaying a fixed amount of enzyme in the presence of varying inhibitor concentrations and low substrate, (cGMP or cAMP in a 3:1 ratio unlabelled to [ $^3$ H]-labeled at a conc  $\sim 1/3 K_m$ ) such that  $IC_{50} \cong K_i$ . The final assay volume was made up to 100  $\mu$ l with assay buffer [20 mM Tris-HCl pH 7.4, 5 mM
- 30  $MgCl_2$ , 1 mg/ml bovine serum albumin]. Reactions were initiated with enzyme, incubated for 30-60 min at 30°C to give <30% substrate turnover and terminated with 50  $\mu$ l yttrium silicate SPA beads (containing 3 mM of the respective unlabelled cyclic nucleotide for PDEs 9 and 11). Plates were re-sealed and shaken for 20 min, after which the beads were allowed to settle for 30 min in the dark and then counted on a
- 35 TopCount plate reader (Packard, Meriden, CT) Radioactivity units were converted to

% activity of an uninhibited control (100%), plotted against inhibitor concentration and inhibitor IC<sub>50</sub> values obtained using the 'Fit Curve' Microsoft Excel extension.

Especially preferred for use herein are compounds which have an IC<sub>50</sub> value of less than about 10, more preferably less than about 5, and most preferably less than about 2nM for the PDE5 enzyme in combination with selectivity of greater than 10-fold, more preferably greater than 50-fold, more preferably greater than 100-fold and especially greater than 200-fold selectivity for the PDE5 enzyme versus the PDE6 enzyme.

#### Functional activity

This can be assessed *in vitro* by determining the capacity of a compound of the invention to enhance sodium nitroprusside-induced relaxation of pre-contracted rabbit corpus cavernosum tissue strips, as described by S.A. Ballard *et al.* (Brit. J. Pharmacol., 1996, 118 (suppl.), abstract 153P).

#### In vivo activity

Compounds were screened in anaesthetised dogs to determine their capacity, after i.v. administration, to enhance the pressure rises in the corpora cavernosa of the penis induced by intracavernosal injection of sodium nitroprusside, using a method based on that described by Trigo-Rocha *et al.* (Neurourol. and Urodyn., 1994, 13, 71).

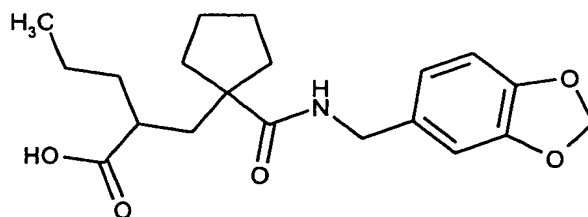
### CHEMICAL COMPOUND EXAMPLES and Preparations

#### NEPi Examples

In addition to the NEPi compounds exemplified herein further NEPi compounds included within the scope of the present invention are as detailed and exemplified in British Patent application GB-A-0101584.1 filed on 22<sup>nd</sup> January 2001 (annex 1 herein).

#### Example 1

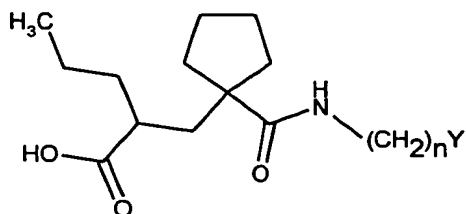
2-({1-[(1,3-Benzodioxol-5-ylamino)carbonyl]cyclopentyl)methyl}pentanoic acid



Trifluoroacetic acid (5ml) was added to a solution of the *tert*-butyl ester from preparation 34 (130mg, 0.31mmol) in dichloromethane (5ml), and the solution stirred at room temperature for 4 hours. The reaction mixture was concentrated under reduced pressure and the residue azeotroped with toluene and dichloromethane to afford the title compound as a clear oil, 112 mg, <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ 0.83 (t, 3H), 1.22-1.40 (m, 3H), 1.50-1.72 (m, 8H), 1.95 (m, 1H), 2.10 (m, 2H), 2.19 (m, 1H), 4.30 (m, 2H), 5.93 (s, 2H), 5.99 (bs, 1H), 6.74 (m, 3H); LRMS: m/z 380 (MH<sup>-</sup>).

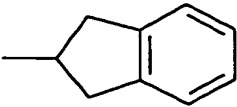
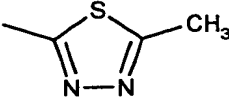
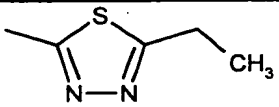
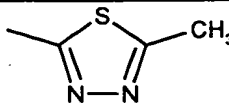
#### 10 Examples 2 to 9

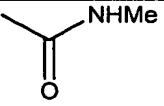
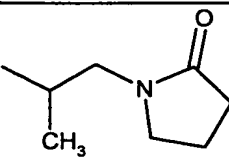
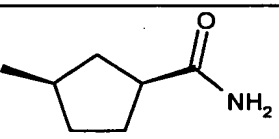
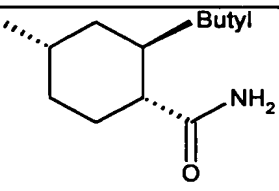
COMPOUNDS OF FORMULA IC, I.E. COMPOUNDS OF GENERAL FORMULA I WHERE R<sup>1</sup> IS PROPYL, WHERE PREPARED FROM THE CORRESPONDING *TERT*-BUTYL ESTER, FOLLOWING A SIMILAR PROCEDURE TO THAT DESCRIBED IN EXAMPLE 1.



(Ic)



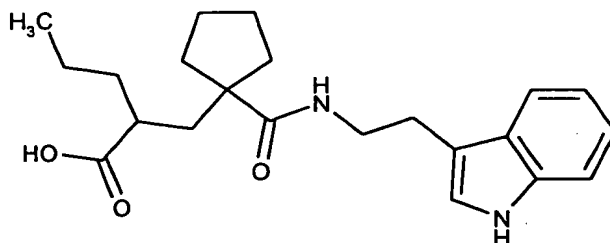
Ex	n	R	Yield	Data
2 <sup>1</sup>	0		78	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) $\delta$ : 0.81 (s, 3H), 1.17-2.04 (m, 14H), 2.27-2.38 (m, 1H), 2.64-2.80 (m, 2H), 3.20-3.31 (m, 2H), 4.60-4.72 (m, 1H), 5.97 (d, 1H), 7.03-7.18 (m, 4H). LRMS : m/z 343.8 (M <sup>+</sup> ).
3 <sup>2,3</sup>	0		81	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) $\delta$ : 0.90 (t, 3H), 1.30-1.42 (m, 4H), 1.59-1.81 (m, 7H), 2.18 (m, 1H), 2.30 (m, 1H), 2.42 (m, 1H), 2.55 (m, 1H), 2.61 (s, 3H). LRMS : m/z 324 (MH <sup>+</sup> ). Mp 184-186°C Anal. Found: C, 55.50; H, 7.22; N, 12.61. C <sub>15</sub> H <sub>23</sub> N <sub>3</sub> O <sub>3</sub> S requires C, 55.36; H, 7.14; N, 12.91%.
4 <sup>3</sup>	0		86	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) $\delta$ : 0.92 (t, 3H), 1.35 (t, 3H), 1.25-1.80 (m, 11H), 2.20-2.50 (m, 4H), 2.95 (q, 2H), 12.10 (bs, 1H). LRMS : m/z 339.8 (MH <sup>+</sup> ) Anal. Found: C, 56.46; H, 7.46; N, 12.36. C <sub>16</sub> H <sub>25</sub> N <sub>3</sub> O <sub>3</sub> S requires C, 56.62; H, 7.44; N, 12.37%.
5 <sup>2</sup>	1		81	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) $\delta$ : 0.80 (t, 3H), 1.20-1.70 (m, 11H), 1.90-2.20 (m, 3H), 2.25 (m, 1H), 2.70 (s, 3H), 4.75 (m, 2H), 7.10 (bs, 1H). LRMS : m/z 340.6 (MH <sup>+</sup> )

Ex	n	R	Yield	Data
6 <sup>2</sup>	2		45	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) $\delta$ : 0.88 (t, 3H), 1.25-1.40 (m, 3H), 1.41-1.70 (m, 8H), 1.92 (m, 1H), 2.00-2.18 (m, 2H), 2.38 (m, 1H), 2.42 (t, 2H), 2.80 (d, 3H), 3.40-3.60 (m, 2H), 6.50 (bs, 1H), 6.74 (bs, 1H). LRMS : m/z 313.2 (MH <sup>+</sup> )
7	0		93	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) $\delta$ : 0.85 (t, 3H), 1.19 (d, 3H), 1.21-1.69 (m, 11H), 1.89-2.10 (m, 5H), 2.30 (m, 1H), 2.41 (m, 2H), 2.95 (m, 1H), 3.35 (m, 1H), 3.63 (m, 2H), 4.20 (m, 1H), 6.58-6.70 (m, 1H). LRMS : m/z 353.1 (MH <sup>+</sup> )
8	0		99	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) $\delta$ : 0.81 (t, 3H), 1.20-1.39 (m, 3H), 1.41-2.10 (m, 1H), 2.80 (m, 1H), 4.35 (m, 17H), 5.81 (d, 1H), 6.30 (bs, 0.5H), 6.43 (bs, 0.5H), 7.40 (bd, 0.5H), 7.61 (bd, 0.5H). LRMS : m/z 339.8 (MH <sup>+</sup> )
9	0			<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) $\delta$ : 0.84 (m, 6H), 1.08-2.08 (m, 29H), 4.29 (m, 1H), 5.95 (d, 1H), 6.43 (s, 1H), 7.80 (d, 1H). LRMS : m/z 409.5 (MH <sup>+</sup> )

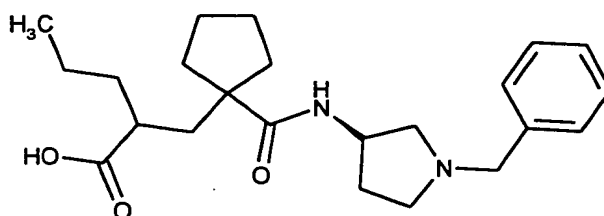
1 = additionally purified by column chromatography on silica gel using ethyl acetate:pentane as eluant.

2 = additionally purified by column chromatography on silica gel using  
5 dichloromethane:methanol as eluant.

3 = recrystallised from ether

Example 102-[1-([2-(1*H*-Indol-3-yl)ethyl]amino)carbonyl]cyclopentyl[methyl]pentanoic acid

Trifluoroacetic acid (2.61ml, 33.9mmol) was added to a solution of the *tert*-butyl ester from preparation 44 (482mg, 1.13mmol) and anisole (1.23ml, 11.3mmol) in dichloromethane (4ml), and the reaction stirred at room temperature for 4 hours. The mixture was washed with water, then brine, dried (MgSO<sub>4</sub>), concentrated under reduced pressure and the residue azeotroped with toluene. The residual brown oil was purified by column chromatography on silica gel using dichloromethane:methanol (95:5) as eluant, and re-columned using an elution gradient of ethyl acetate:pentane (30:70 to 50:50) to afford the title compound as a clear foam, 136mg, 32%; <sup>1</sup>HNMR (CDCl<sub>3</sub>, 400MHz) δ: 0.82 (s, 3H), 1.16-1.77 (m, 12H), 1.78-2.03 (m, 2H), 2.36 (m, 1H), 2.97 (m, 2H), 3.61 (m, 2H), 5.83 (m, 1H), 7.04 (s, 1H), 7.09-7.23 (m, 2H), 7.39 (d, 1H), 7.61 (d, 1H), 8.15 (m, 1H); LRMS : m/z 371.8 (MH<sup>+</sup>).

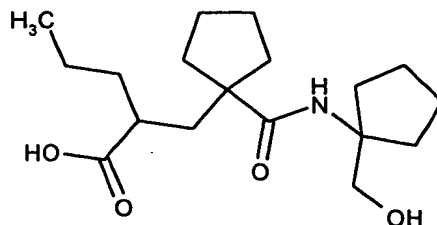
Example 112-[1-([(3*S*)-1-Benzylpyrrolidinyl]amino)carbonyl]cyclopentyl[methyl]pentanoic acid

A solution of the *tert*-butyl ester from preparation 45 (70mg, 0.16mmol) in trifluoroacetic acid (1ml) and dichloromethane (1ml) was stirred at room temperature for 2 hours. The reaction was concentrated under reduced pressure and the residue azeotroped with dichloromethane. The residue was partitioned between water (1ml) and ethyl acetate (5ml), and the pH of the aqueous layer adjusted to 6 using sodium bicarbonate solution. The layers were separated, the organic phase dried (Na<sub>2</sub>SO<sub>4</sub>), evaporated under reduced pressure and the residue azeotroped with dichloromethane, to give the title compound as a beige foam, 45mg, 73%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.84 (t, 3H), 1.20-2.95 (m, 19H), 3.52 (m, 1H), 3.75 (m, 1H), 3.95

(m, 1H), 4.25 (m, 1H), 4.45 (m, 1H), 6.96 (bs, 1H), 7.39 (m, 5H); LRMS : m/z 387 (MH<sup>+</sup>); Anal. Found: C, 61.11; H, 7.69; N, 6.00. C<sub>23</sub>H<sub>34</sub>N<sub>2</sub>O<sub>3</sub>·CH<sub>2</sub>Cl<sub>2</sub> requires C, 61.14; H, 7.70; N, 5.94%.

### 5 Example 12

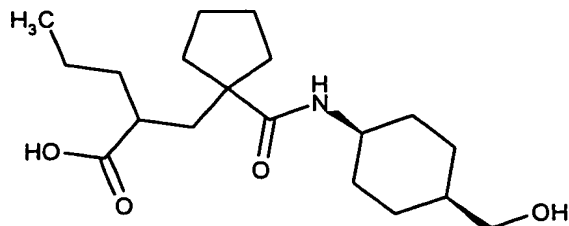
2-([1-([1-(Hydroxymethyl)cyclopentyl]amino)carbonyl]cyclopentyl)methyl]pentanoic acid



- 10 A solution of the *tert*-butyl ester from preparation 33 (38mg, 0.1mmol) in trifluoroacetic acid (2ml) and dichloromethane (2ml) was stirred at room temperature for 2 hours. The reaction was concentrated under reduced pressure and the residue azeotroped with toluene and then dichloromethane to give a colourless gum. This was suspended in a solution of potassium carbonate (50mg, 0.3mmol) in methanol, and the mixture stirred for 2 hours at room temperature. The methanol was removed under reduced pressure, the residual aqueous mixture diluted with water (20ml), and acidified to pH 2 using 2N hydrochloric acid. This solution was extracted with ethyl acetate (2x20ml), and the combined organic solutions dried (MgSO<sub>4</sub>), and evaporated under reduced pressure to give a clear oil, 32mg, 97%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ:: 0.88 (t, 3H), 1.20-1.40 (m, 3H), 1.41-1.90 (m, 17H), 2.01-2.20 (m, 2H), 2.40 (m, 1H), 3.71 (dd, 2H), 5.80 (bs, 1H); LRMS : m/z 326.1 (MH<sup>+</sup>)

### Example 13

Cis-2-([1-([4-(Hydroxymethyl)cyclohexyl]amino)carbonyl]cyclopentyl)methyl]pentanoic acid



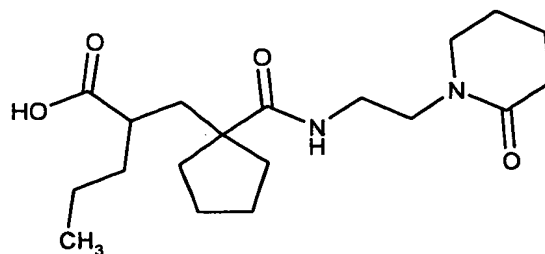
The title compound was obtained as a colourless gum in 68%, from the *tert*-butyl ester from preparation 43, following the procedure described in example 12, except

the product was additionally purified by column chromatography on silica gel using dichloromethane:methanol (95:5) as the eluant;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz)  $\delta$ : 0.87 (t, 3H), 1.21-1.40 (m, 6H), 1.52-1.70 (m, 15H), 1.92-2.11 (m, 3H), 2.39 (m, 1H), 3.55 (d, 2H), 4.01 (m, 1H), 5.90 (m, 1H); LRMS :  $m/z$  340.3 ( $\text{MH}^+$ ).

5

#### Example 14

#### 2-([1-([2-(2-Oxo-1-piperidinyl)ethyl]amino)carbonyl]cyclopentyl)methyl]pentanoic acid

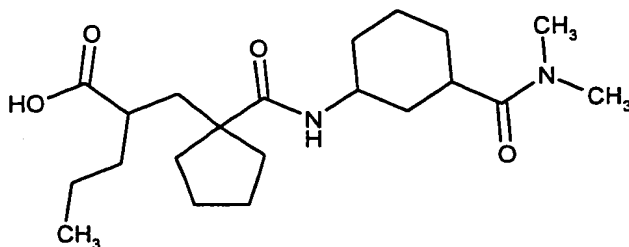


- 10 Hydrogen chloride gas was bubbled through an ice-cold solution of the *tert*-butyl ester from preparation 47 (43mg, 0.105mmol) in dichloromethane (10ml), for 20 minutes. The solution was then stirred at room temperature for 3 hours. The mixture was concentrated under reduced pressure and the residue azeotroped with dichloromethane (3x), to give a glass-like solid. The crude product was purified by
- 15 column chromatography on silica gel using an elution gradient of dichloromethane:methanol (95:5 to 90:10) to afford the title compound, 6mg;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz)  $\delta$ : 0.81 (t, 3H), 1.20-1.36 (m, 4H), 1.41-1.69 (m, 7H), 1.79 (m, 4H), 1.90-2.10 (m, 3H), 2.30 (m, 1H), 2.38 (t, 2H), 3.30-3.60 (m, 6H), 7.00 (bs, 1H); LRMS :  $m/z$  351 ( $\text{M-H}^-$ ).

20

#### Example 15

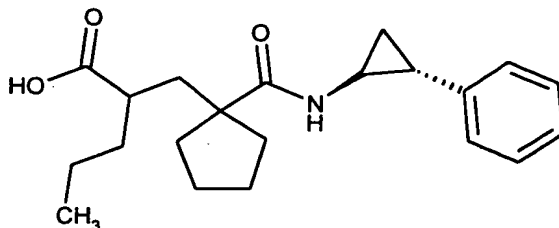
#### 2-([1-([3-[(Dimethylamino)carbonyl]cyclohexyl]amino)carbonyl]cyclopentyl)methyl]pentanoic acid



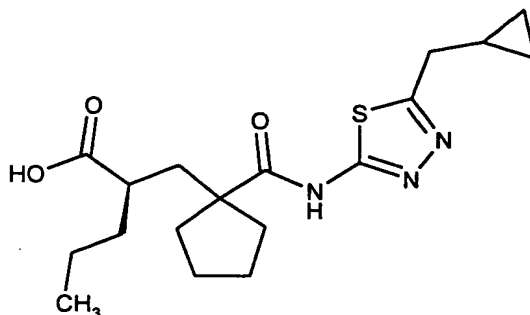
- 25 The title compound was obtained as a solid in 85% yield, from the *tert*-butyl ester from preparation 42, following a similar method to that described in example 14, except that dichloromethane:methanol:acetic acid (95:3:2) was used as the

chromatographic eluant;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz)  $\delta$ : 0.89 (t, 3H), 1.09-1.76 (m, 12H), 1.80-2.17 (m, 10H), 2.37 (m, 1H), 2.68 (m, 1H), 2.95 (s, 3H), 3.04 (s, 3H), 3.83 (m, 1H), 6.06 (m, 1H); LRMS :  $m/z$  381 ( $\text{MH}^+$ ); Anal. Found: C, 63.31; H, 9.17; N, 6.53.  $\text{C}_{21}\text{H}_{36}\text{N}_2\text{O}_4 \cdot \text{H}_2\text{O}$  requires C, 63.29; H, 9.61; N, 7.03%.

5

**Example 16****2-([1-((1*R*,2*R*)-2-Phenylcyclopropyl)amino)carbonyl)cyclopentyl]methyl}pentanoic acid**

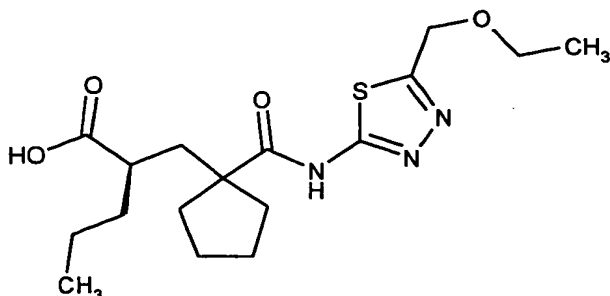
- 10 The title compound was obtained quantitatively as an orange gum from the *tert*-butyl ester from preparation 46, following a similar procedure to that described in example 14;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz)  $\delta$ : 0.90 (t, 3H), 1.12-2.14 (m, 17H), 2.38 (m, 1H), 2.87 (m, 1H), 6.10 (s, 1H), 7.13 (m, 3H), 7.25 (m, 2H); LRMS :  $m/z$  344.3 ( $\text{MH}^+$ ).

**Example 17****(2*R*)-2-([1-([5-(Cyclopropylmethyl)-1,3,4-thiadiazol-2-yl]amino)carbonyl)cyclopentyl]methyl}pentanoic acid**

- A solution of the *tert*-butyl ester from preparation 50 (63mg, 0.15mmol) in trifluoroacetic acid (2ml) and dichloromethane (2ml), was stirred at room temperature for 2 hours. The mixture was concentrated under reduced pressure and the residue purified by column chromatography on silica gel using dichloromethane:methanol (95:5) as eluant, to give the title compound as a white foam, 46mg, 83%;  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ , 400MHz)  $\delta$ : 0.38 (m, 2H), 0.62 (m, 2H), 0.82 (t, 3H), 1.12 (m, 1H), 1.26 (m, 2H), 1.38 (m, 1H), 1.52 (m, 1H), 1.78-1.78 (m, 6H), 1.90 (m, 1H), 2.23 (m, 4H), 2.92 (d, 2H); LRMS :  $m/z$  366.0 ( $\text{MH}^+$ );  $[\alpha]_D = -7.75^\circ$  ( $c = 0.08$ , methanol).

Example 18

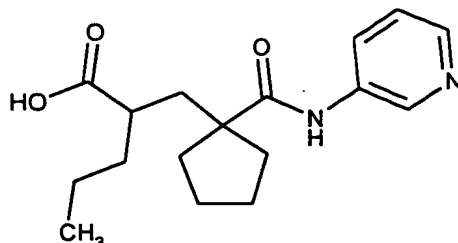
(2R)-2-([1-([5-(Ethoxymethyl)-1,3,4-thiadiazol-2-yl]amino)carbonyl]cyclopentyl)-methyl]pentanoic acid



The title compound was obtained as a white foam in 62% yield, from the *tert*-butyl ester from preparation 51, following a similar procedure to that described in example 17;  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ , 400MHz)  $\delta$ : 0.82 (t, 3H), 1.21-1.40 (m, 7H), 1.50 (m, 1H), 1.60-1.77 (m, 7H), 1.88 (m, 1H), 2.23 (m, 4H), 3.62 (q, 2H);  $[\alpha]_D = -6.08^\circ$  ( $c = 0.25$ , methanol).

Example 19

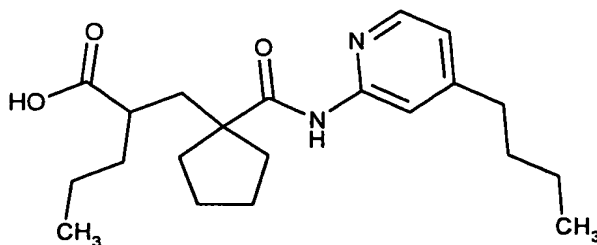
2-([1-([3-Pyridinylamino)carbonyl]cyclopentyl)methyl]pentanoic acid



A mixture of the benzyl ester from preparation 52 (130mg, 0.33mmol) and 10% palladium on charcoal (20mg) in 95% aqueous ethanol (3ml) was hydrogenated at 15psi and room temperature for 2 hours. The reaction was filtered through Arbocel®, washing through with ethanol, and the filtrate evaporated under reduced pressure. The residual gum was purified by column chromatography on silica gel using dichloromethane:methanol (95:5) as eluant to afford the title compound, 103mg, 83%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz)  $\delta$ : 0.90 (t, 3H), 1.38 (m, 2H), 1.44 (m, 1H), 1.58-1.82 (m, 8H), 2.19 (m, 1H), 2.39 (m, 2H), 2.52 (m, 1H), 6.88 (m, 1H), 7.67 (m, 1H), 7.82 (d, 1H), 8.38 (d, 1H), 9.78 (s, 1H); LRMS :  $m/z$  305 ( $\text{MH}^+$ ).

Example 20

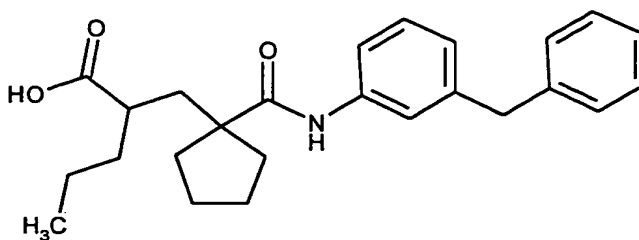
2-[(1-[(4-Butyl-2-pyridinyl)amino]carbonyl)cyclopentyl)methyl]pentanoic acid



The title compound was obtained in 92% yield from the benzyl ester from preparation 55, following a similar procedure to that described in example 19; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.90 (m, 6H), 1.28-1.50 (m, 5H), 1.58-1.81 (m, 10H), 2.20 (m, 1H), 2.40 (m, 2H), 2.58 (m, 3H), 6.70 (d, 1H), 7.68 (d, 1H), 8.22 (s, 1H), 9.90 (bs, 1H).

Example 21

2-[(1-[(3-Benzylanilino)carbonyl)cyclopentyl)methyl]pentanoic acid



10

A mixture of the benzyl ester from preparation 53 (1.3mg, 2.47mmol) and 5% palladium on charcoal (130mg) in water (10ml) and ethanol (40ml) was hydrogenated at 30 psi and room temperature for 2 hours. The reaction mixture was filtered through Arbocel®, the filtrate concentrated under reduced pressure, and the residue trituated with dichloromethane. The residual gum was trituated with ether, then hexane, and dried at 50°C, to give the title compound as a solid, 0.79g, 81%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.95 (t, 3H), 1.24-1.51 (m, 3H), 1.58-1.80 (m, 7H), 1.88 (dd, 1H), 2.15 (m, 2H), 2.24 (m, 1H), 2.48 (m, 1H), 4.00 (s, 2H), 6.98 (d, 1H), 7.24 (m, 6H), 7.40 (m, 3H); Anal. Found: C, 75.48; H, 7.76; N, 3.59. C<sub>25</sub>H<sub>31</sub>NO<sub>3</sub>·0.25H<sub>2</sub>O requires C, 75.44; H, 7.98; N, 3.51%.

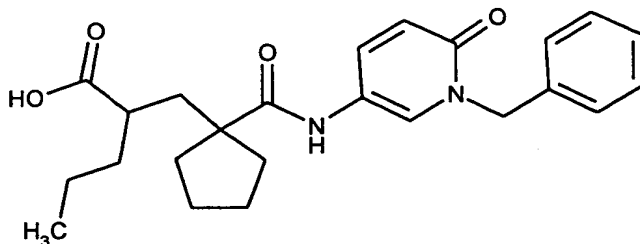
15

20



Example 22

2-[(1-[(1-Benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl)-cyclopentyl)methyl]-pentanoic acid.



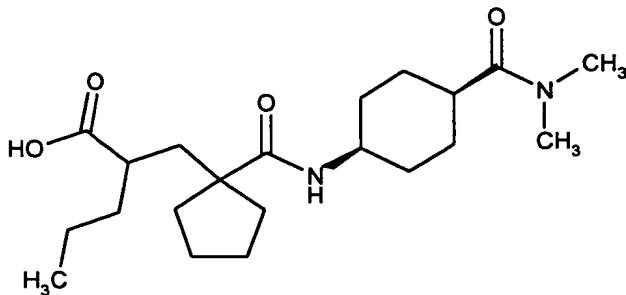
5

The title compound was obtained as a white foam in 51% yield from the benzyl ester from preparation 56, following a similar procedure to that described in example 21, except, the product was purified by column chromatography on silica gel, using ethyl acetate as eluant; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.96 (t, 3H), 1.28-1.80 (m, 12H), 2.01 (m, 1H), 2.30-2.52 (m, 2H), 5.02 (dd, 2H), 6.60 (d, 1H), 7.27 (m, 5H), 7.70 (s, 1H), 8.34 (s, 1H); Anal. Found: C, 69.52; H, 7.41; N, 6.51. C<sub>24</sub>H<sub>30</sub>N<sub>2</sub>O<sub>4</sub>·0.25H<sub>2</sub>O requires C, 69.45; H, 7.41; N, 6.75.

10

Example 23

Cis-2-[(1-[(4-[(Dimethylamino)carbonyl]cyclohexyl)amino]carbonyl)-cyclopentyl)methyl]pentanoic acid



A mixture of the benzyl ester from preparation 58 (150mg, 0.33mmol) and 10% palladium on charcoal (20mg) in water (0.3ml) and ethanol (3.5ml) was hydrogenated at 15 psi and room temperature for 3 days. The reaction mixture was filtered through Arbocel®, and the filtrate concentrated under reduced pressure. The residual gum was purified by column chromatography on silica gel using dichloromethane:methanol (95:5) as eluant to afford the title compound, 85mg, 65%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.84 (t, 3H), 1.29-1.96 (m, 18H), 2.01-2.23 (m, 4H), 2.37 (m, 1H), 2.62 (m, 1H), 2.96 (s, 3H), 3.03 (s, 3H), 3.96 (m, 1H), 5.98 (m, 1H); LRMS :

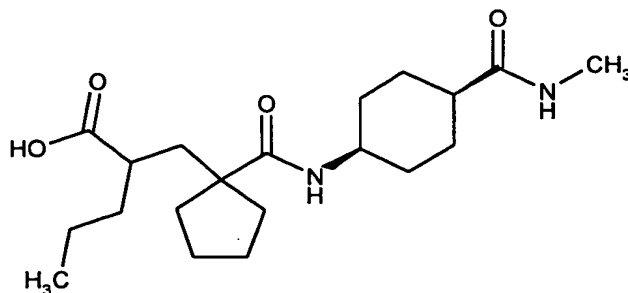
20

25

m/z 381.8 (MH<sup>+</sup>); Anal. Found: C, 63.81; H, 9.58; N, 6.99. C<sub>21</sub>H<sub>36</sub>N<sub>2</sub>O<sub>4</sub>·0.2CH<sub>2</sub>Cl<sub>2</sub> requires C, 64.06; H, 9.23; N, 7.05%.

#### Example 24

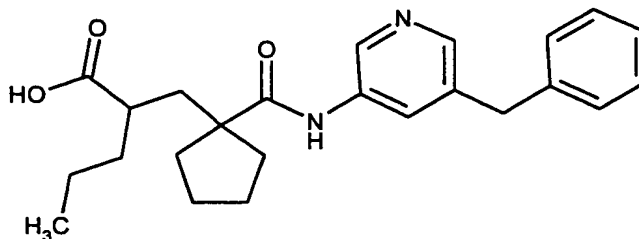
- 5 Cis-2-({1-[(4-[(Methylamino)carbonyl]cyclohexyl)amino]carbonyl]cyclopentyl}-methyl)pentanoic acid



- The title compound was obtained as a white solid in 34% yield from the benzyl ester from preparation 59, following the procedure described in example 23; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.90 (t, 3H), 1.26-2.02 (m, 20H), 2.19 (m, 3H), 2.39 (m, 1H), 2.82 (d, 3H), 4.00 (m, 1H), 5.69 (m, 1H), 6.00 (d, 1H); LRMS : m/z 365 (M-H<sup>-</sup>).

#### Example 25

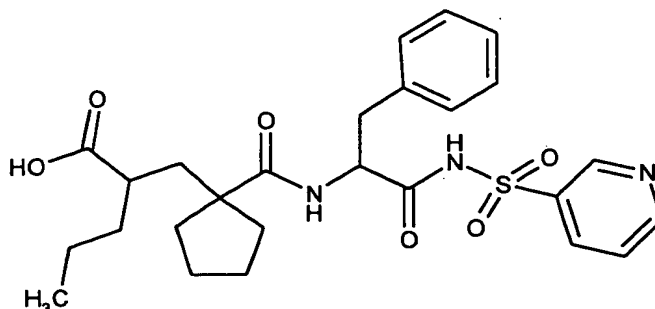
- 15 2-[(1-[(5-Benzyl-3-pyridinyl)amino]carbonyl]cyclopentyl)methyl]-pentanoic acid.



- A mixture of the benzyl ester from preparation 54 (850mg, 1.76mmol) and 5% palladium on charcoal (100mg) in 20% aqueous ethanol (30ml) was hydrogenated at 30 psi and room temperature for 2 hours. The mixture was filtered through Arbocel®, the filtrate evaporated under reduced pressure, and the residue azeotroped with dichloromethane to give the title compound as a foam, 0.63g; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.92 (t, 3H), 1.30-1.83 (m, 11H), 2.07 (m, 1H), 2.42 (m, 3H), 3.82 (s, 2H), 7.15-7.38 (5H), 7.80 (s, 1H), 8.48 (s, 1H), 8.59 (s, 1H), 8.62 (s, 1H); Anal. Found: C, 72.29; H, 7.70; N, 6.90. C<sub>24</sub>H<sub>30</sub>N<sub>2</sub>O<sub>3</sub>·0.25H<sub>2</sub>O requires C, 72.24; H, 7.70; N, 7.02%.

Example 26

2-({1-[({1-Benzyl-2-oxo-2-[3-pyridinylsulfonyl]amino]ethyl}amino)-carbonyl]cyclopentyl}methyl)pentanoic acid.



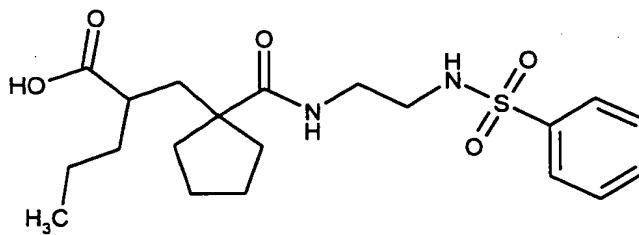
5

A mixture of the benzyl ester from preparation 57 (918mg, 1.52mmol) and 10% palladium on charcoal (90mg) in water (10ml) and ethanol (50ml) was hydrogenated at 50 psi and room temperature for 4 ½ hours. Tlc analysis showed starting material remaining, so additional catalyst (70mg) was added, and the mixture hydrogenated for a further 18 hours. Tlc analysis, again showed starting material remaining, so further catalyst (70mg) was added, and hydrogenation continued for an additional 6 hours. The reaction mixture was filtered through Arbocel®, the filtrate evaporated under reduced pressure and the residue azeotroped with dichloromethane. The crude product was purified by column chromatography on silica gel using an elution gradient of dichloromethane:acetic acid:ethanol (99:1:0 to 79:1:0.9:20) to afford the title compound as a white foam, 271mg, 35%; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300MHz) δ: 0.75 (m, 3H), 0.96-1.42 (m, 11H), 1.61-1.99 (m, 4H), 2.75-3.02 (m, 2H), 4.45 (m, 1H), 7.20 (m, 6H), 7.62 (m, 1H), 8.24 (m, 1H), 8.83 (s, 1H), 9.01 (s, 1H), 11.98 (bs, 1H), 12.70 (bs, 1H); IR (KBr disc) 1185, 1195 (m), 1455, 1515, 1640, 1704, 2870, 2930, 2960 (s).

20

Example 27

2-({1-[({2-[Phenylsulfonyl]amino]ethyl}amino)carbonyl]cyclopentyl}methyl)pentanoic acid

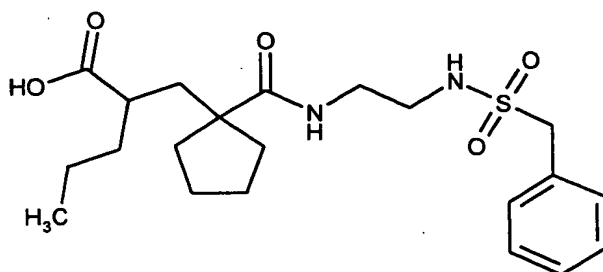


25

A mixture of the amine from preparation 61 (235mg, 0.72mmol), benzenesulphonyl chloride (127mg, 0.72mmol) and triethylamine (150 $\mu$ l, 1.08mmol) in dichloromethane (6ml) was stirred at room temperature for 2 days. The mixture was concentrated under reduced pressure and the residue purified by column chromatography on silica gel using ethyl acetate:pentane (30:70) as eluant to give a clear oil. This was then dissolved in trifluoroacetic acid (3ml) and dichloromethane (3ml) and the solution stirred at room temperature for 6 hours. The mixture was concentrated under reduced pressure and the residue azeotroped twice with toluene. The crude product was purified by column chromatography on silica gel using ethyl acetate:pentane (30:70) to afford the title compound as a clear oil, 204mg, 69%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz)  $\delta$ : 0.84 (t, 3H), 1.22-1.43 (m, 4H), 1.43-2.18 (m, 10H), 2.36 (m, 1H), 3.11 (m, 2H), 3.20-3.31 (m, 1H), 3.42-3.53 (m, 1H), 6.13-6.24 (m, 1H), 7.42-7.59 (m, 3H), 7.84 (m, 2H); LRMS : m/z 411.8 ( $\text{MH}^+$ ); Anal. Found: C, 57.26; H, 7.40; N, 6.61.  $\text{C}_{20}\text{H}_{30}\text{N}_2\text{O}_5\text{S}$  requires C, 57.18; H, 7.22; N, 6.62%.

#### Example 28

2-({1-[(2-[(Benzylsulfonyl)amino]ethyl)amino]carbonyl]cyclopentyl)methyl}pentanoic acid



The title compound was obtained as a clear oil in 97% yield, from the amine from preparation 61, following the procedure described in example 27,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300MHz)  $\delta$ : 0.87 (t, 3H), 1.19-1.72 (m, 11H), 1.80-1.96 (m, 1H), 2.00-2.16 (m, 2H), 2.27-2.38 (m, 1H), 2.92-3.21 (m, 3H), 3.23-3.39 (m, 1H), 4.25 (s, 2H), 5.80-6.06 (m, 1H), 6.38 (m, 1H), 7.29-7.43 (m, 5H); LRMS : m/z 425.8 ( $\text{MH}^+$ ).

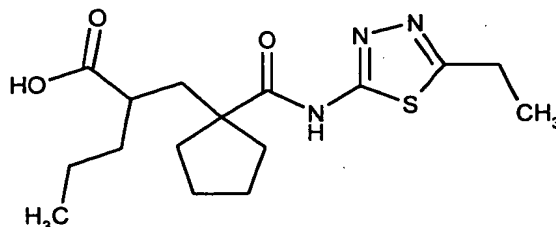
Example 29

(-)-2-[1-[(5-Ethyl-1,3,4-thiadiazol-2-yl)amino]carbonyl]cyclopentyl)methyl]pentanoic acid

and

5 Example 30

(+)-2-[1-[(5-Ethyl-1,3,4-thiadiazol-2-yl)amino]carbonyl]cyclopentyl)methyl]pentanoic acid



The acid from Example 4 (824mg) was further purified by HPLC using an AD column and using hexane:iso-propanol:trifluoroacetic acid (85:15:0.2) as eluant to give the title compound of example 29 as a white foam, 400mg, 99.5% ee, <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.90 (t, 3H), 1.36 (m, 6H), 1.50-1.80 (m, 9H), 2.19 (m, 1H), 2.30 (m, 1H), 2.44 (m, 1H), 2.60 (m, 1H), 2.98 (q, 2H), 12.10-12.30 (bs, 1H), LRMS : m/z 338 (MH<sup>+</sup>), [α]<sub>D</sub> = -9.0° (c = 0.1, methanol), and the title compound of example 30 as a white foam, 386mg, 99% ee, <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.90 (t, 3H), 1.38 (m, 6H), 1.50-1.79 (m, 9H), 2.19 (m, 1H), 2.30 (m, 1H), 2.44 (m, 1H), 2.60 (m, 1H), 2.98 (q, 2H), 12.10-12.27 (bs, 1H); LRMS: m/z 338 (MH<sup>+</sup>); and [α]<sub>D</sub> = +3.8° (c = 0.1, methanol)

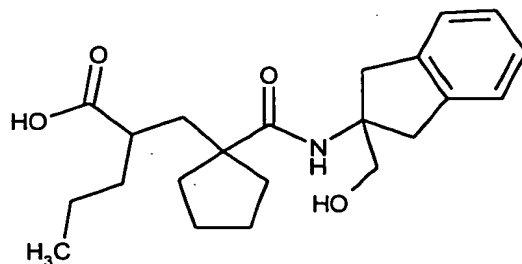
Example 31

(+)-2-([1-([2-(Hydroxymethyl)-2,3-dihydro-1H-inden-2-yl]amino)carbonyl]cyclopentyl)-methyl]pentanoic acid

and

5 Example 32

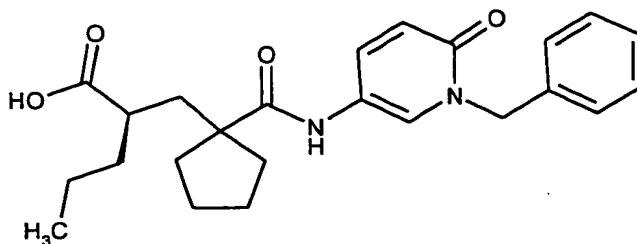
(-)-2-([1-([2-(Hydroxymethyl)-2,3-dihydro-1H-inden-2-yl]amino)carbonyl]cyclopentyl)-methyl]pentanoic acid



2-([1-([2-(Hydroxymethyl)-2,3-dihydro-1H-inden-2-yl]amino)carbonyl]cyclopentyl)-methyl]pentanoic acid (WO 9110644) was further purified by HPLC using an AD column and hexane:isopropanol:trifluoroacetic acid (90:10:0.1) as eluant, to give the title compound of example 31, 99% ee,  $[\alpha]_D = +10.4^\circ$  (c = 0.067, ethanol) and the title compound of example 32, 99% ee,  $[\alpha]_D = -10.9^\circ$  (c = 0.046, ethanol).

15 Example 33

(2R)-2-([1-([1-(1-Benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl]cyclopentyl)methyl]-pentanoic acid.

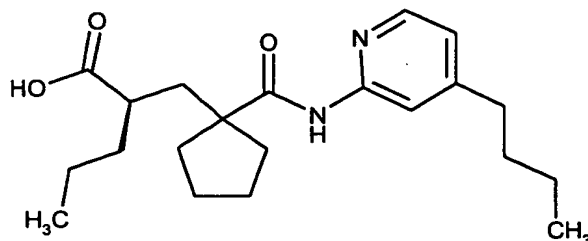


1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (191mg, 1.0mmol), 1-hydroxybenzotriazole hydrate (135mg, 01.0mmol), N-methylmorpholine (165μl, 1.5mmol) and finally the amine from preparation 28 (150mg, 0.69mmol) were added to a solution of the acid from preparation 2 (284mg, 1.0mmol) in N,N-dimethylformamide (8ml), and the reaction stirred at 90°C for 18 hours. The cooled solution was diluted with ethyl acetate (90ml), washed with water (4x50ml), and brine (50ml), then dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The crude product was purified by chromatography on silica gel, using ethyl acetate:pentane

(30:70) to give a yellow oil, 191mg. This intermediate was dissolved in dichloromethane (3ml) and trifluoroacetic acid (3ml) and the solution stirred at room temperature for 5 hours. The mixture was concentrated under reduced pressure and the residue purified by column chromatography on silica gel using dichloromethane:methanol (95:5) as eluant to give the title compound as a foam, 77mg,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300MHz)  $\delta$ : 0.86 (t, 3H), 1.20-1.76 (m, 12H), 1.93-2.02 (m, 1H), 2.20-2.46 (m, 3H), 4.95 (d, 1H), 5.04 (d, 1H), 6.61 (d, 1H), 7.21 (m, 1H), 7.50 (s, 1H), 8.23 (s, 1H); LRMS : m/z 411.6 (MH) $^+$ ;  $[\alpha]_D = -3.8^\circ$  (c = 0.052, ethanol).

#### 10 Example 34

(2R)-2-[(1-[(4-Butyl-2-pyridinyl)amino]carbonyl)cyclopentyl)methyl]pentanoic acid

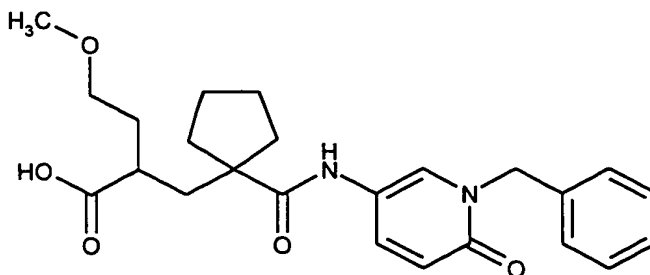


The title compound was obtained in 43% yield from the acid from preparation 2 and the amine from preparation 30, following a similar procedure to that described in example 33,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz)  $\delta$ : 0.80-1.00 (m, 6H), 1.22-1.84 (m, 18H), 2.03-2.56 (m, 3H), 2.77 (m, 1H), 7.14 (d, 1H), 8.08 (d, 1H), 8.23 (s, 1H), 11.71 (brs, 1H).

LRMS : m/z 361.7 (MH) $^+$ ,  $[\alpha]_D = -1.4^\circ$  (c = 0.14, ethanol).

#### 20 Example 35

2-[(1-[(1-Benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl)cyclopentyl)methyl]-4-methoxybutanoic acid



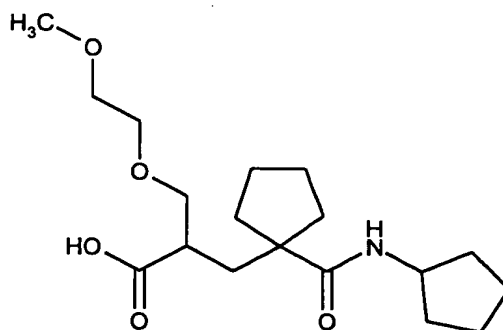
A mixture of the benzyl ester from preparation 62 (850mg, 1.64mmol), and 5% palladium on charcoal (250mg) in 40% aqueous ethanol (21ml), was hydrogenated at 30 psi and room temperature for 30 minutes. The reaction mixture was filtered

through Hyflo®, and the filtrate evaporated under reduced pressure. The residual foam was purified by column chromatography on silica gel using dichloromethane:methanol (97:3) as eluant to give the title compound as a white foam, 550mg, 79%; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300MHz) δ: 1.24-2.17 (m, 12H), 2.18-2.31 (m, 1H), 3.07 (s, 3H), 3.21 (t, 2H), 5.08 (s, 2H), 6.63 (d, 1H), 7.23-7.41 (m, 5H), 7.72 (d, 1H), 8.24 (s, 1H).

Anal. Found: C, 67.46; H, 7.18; N, 6.24. C<sub>24</sub>H<sub>30</sub>N<sub>2</sub>O<sub>5</sub> requires C, 67.58; H, 7.09; N, 6.57%.

10 **Example 36**

3-{1-[(Cyclopentylamino)carbonyl]cyclopentyl}-2-[(2-methoxyethoxy)methyl]propanoic acid

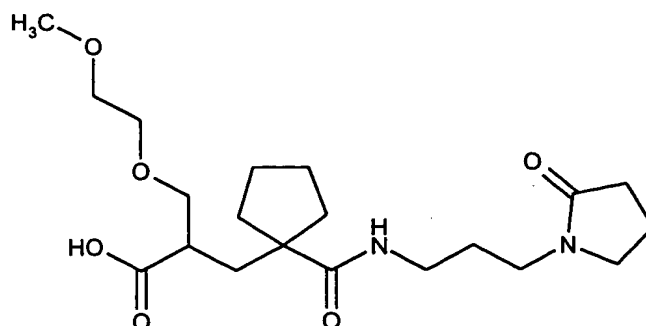


A solution of the *tert*-butyl ester from preparation 64 (320mg, 0.80mmol) in trifluoroacetic acid (2ml) and dichloromethane (2ml) was stirred at room temperature for 8 hours. The mixture was concentrated under reduced pressure and the residue azeotropeed twice with toluene. The crude product was purified by column chromatography on silica gel using dichloromethane:methanol (95:5) to give the title compound as a clear oil, 171mg, 62%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.29-1.40 (m, 2H), 1.42-1.69 (m, 10H), 1.75 (dd, 1H), 1.87-2.03 (m, 5H), 2.64 (m, 1H), 3.34 (s, 3H), 3.43-3.52 (m, 3H), 3.57 (m, 2H), 3.61 (m, 1H), 4.08-4.20 (m, 1H), 5.89 (d, 1H); LRMS : m/z 340 (MH<sup>+</sup>).



Example 37

3-(2-Methoxyethoxy)-2-[[1-([3-(2-oxo-1-pyrrolidiny)propyl]amino)carbonyl]-cyclopentyl]methyl]propanoic acid

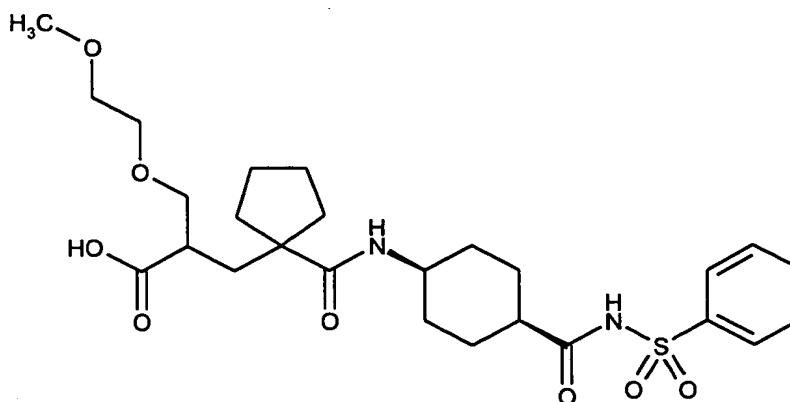


- 5 The title compound was obtained as a clear oil in 57% yield from the *tert*-butyl ester of preparation 65, following the procedure described in example 36,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300MHz)  $\delta$ : 1.56-1.78 (m, 8H), 1.94-2.17 (m, 6H), 2.44 (m, 2H), 2.68-2.76 (m, 1H), 3.10-3.21 (m, 1H), 3.22-3.31 (m, 1H), 3.37 (s, 3H), 3.40 (m, 2H), 3.44-3.56 (m, 5H), 3.60 (m, 2H), 3.68 (m, 1H), 6.91-7.01 (m, 1H); LRMS :  $m/z$  398.7 ( $\text{M}^+$ )

10

Example 38

Cis-3-(2-Methoxyethoxy)-2-[[1-[(4-[(phenylsulfonyl)amino]carbonyl]cyclohexyl)-amino]carbonyl]cyclopentyl]methyl]propanoic acid

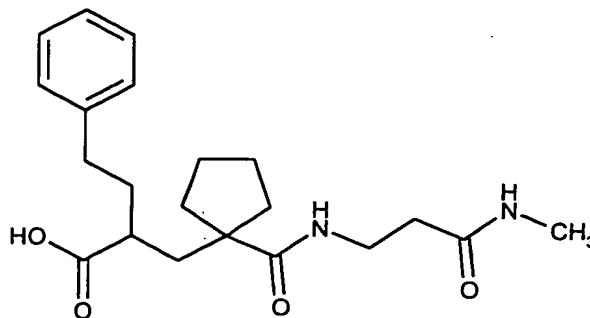


- 15 A solution of the *tert*-butyl ester from preparation 66 (446mg, 0.75mmol) in dichloromethane (5ml) and trifluoroacetic acid (5ml) was stirred at room temperature for 18 hours. The reaction mixture was concentrated under reduced pressure, and the residue azeotroped with dichloromethane, then toluene, and finally ether, to afford the title compound as a white foam, 385mg, 95%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz)  $\delta$ :  
 20 1.48-2.17 (m, 18H), 2.40 (s, 1H), 2.66 (s, 1H), 3.37 (s, 3H), 3.50-3.70 (m, 6H), 3.94 (s, 1H), 6.10 (d, 1H), 6.59 (s, 1H), 7.55 (t, 2H), 7.61 (m, 1H), 8.02 (d, 2H), 9.11 (s,

1H); Anal. Found: C, 54.88; H, 6.90; N, 5.04.  $C_{26}H_{38}N_2O_8S \cdot 1.7H_2O$  requires C, 57.97; H, 7.11; N, 5.20%.

### Example 39

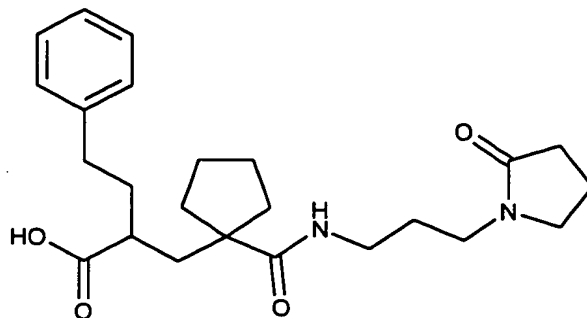
5 2-{{1-({3-(Methylamino)-3-oxopropyl}amino)carbonyl}cyclopentyl[methyl]}-4-phenylbutanoic acid



A mixture of the benzyl ester from preparation 68 (160mg, 0.34mmol) and 10% palladium on charcoal (100mg) in ethanol (30ml) was hydrogenated at room  
10 temperature and 60 psi for 18 hours. The mixture was filtered through Arbocel® and the filtrate concentrated under reduced pressure, and azeotroped with dichloromethane. The crude product was purified by column chromatography on silica gel using an elution gradient of dichloromethane:methanol:acetic acid (95:5:0 to 95:5:0.5) to afford the title compound as a white foam, 100mg, 79%;  $^1H$  NMR ( $CDCl_3$ ,  
15 400MHz)  $\delta$ : 1.40-1.70 (m, 8H), 1.95 (m, 3H), 2.10 (m, 1H), 2.35 (d, 3H), 2.59 (m, 2H), 2.75 (t, 3H), 3.42 (m, 2H), 6.25 (bs, 1H), 6.70 (bs, 1H), 7.13-7.25 (m, 5H); and LRMS: m/z 375.0 ( $MH^+$ ).

### Example 40

20 2-{{1-({3-(2-Oxo-1-pyrrolidinyl)propyl}amino)carbonyl}cyclopentyl]-methyl}-4-phenylbutanoic acid.

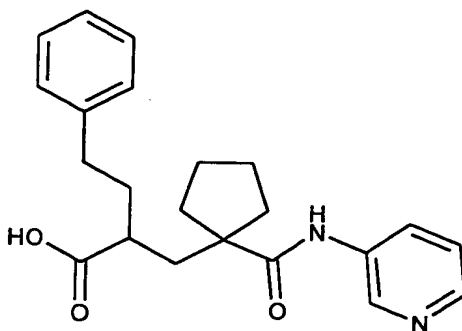


A mixture of the benzyl ester from preparation 67 (780mg, 1.55mmol) and 10% palladium on charcoal (100mg) in ethanol:water (90:10 by volume), (30ml) was

hydrogenated at room temperature under 60psi H<sub>2</sub> pressure for 1.5 hours. The catalyst was filtered off, and the filtrate evaporated under reduced pressure to provide the title compound as a white foam, 473mg, 74%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ : 1.26-1.77 (m, 10H), 1.78-2.46 (m, 11H), 2.49-2.70 (m, 2H), 2.95-3.36 (m, 4H), 6.92-7.38 (m, 5H); Anal. Found: C, 64.05; H, 7.73; N, 6.22. C<sub>24</sub>H<sub>34</sub>N<sub>2</sub>O<sub>4</sub>·0.75H<sub>2</sub>O requires C, 65.88; H, 7.83; N, 6.40%.

#### Example 41

##### 4-Phenyl-2-({1-[(3-pyridinylamino)carbonyl]cyclopentyl}methyl)butanoic acid



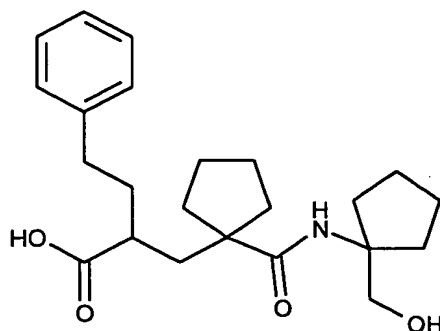
10

A mixture of the benzyl ester from preparation 71 (700mg, 1.53mmol) and 5% palladium on charcoal (70mg) in ethanol:water (90:10 by volume, 50ml) was hydrogenated at room temperature under 30 psi H<sub>2</sub> pressure for 5 hours. The catalyst was filtered through Arbocel®, washing well with ethanol, and the filtrate evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane:methanol (95:5) as the eluant to provide the title compound as a white foam, 510mg, 91%; mp 80-85°C (collapses to a gum); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 1.40-2.78 (m, 15H), 6.93-7.39 (m, 5H), 7.93 (m, 1H), 8.59 (d, 1H), 9.17 (d, 1H), 9.41 (s, 1H); Anal. Found: C, 70.83; H, 7.10; N, 7.64. C<sub>22</sub>H<sub>26</sub>N<sub>2</sub>O<sub>3</sub>·0.3H<sub>2</sub>O requires C, 70.94; H, 7.22; N, 7.52%.

20

Example 42

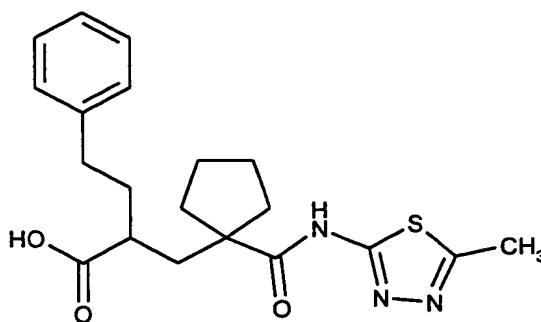
2-[[1-[[1-(Hydroxymethyl)cyclopentyl]amino]carbonyl]cyclopentyl]methyl]-4-phenylbutanoic acid



- 5 A mixture of the benzyl ester from preparation 69 (118mg, 0.25mmol) and 10% palladium on charcoal (100mg) in ethanol (20ml) was hydrogenated at room temperature and 60 psi for 18 hours. The mixture was filtered through Arbocel®, the filtrate concentrated under reduced pressure, and azeotroped with dichloromethane to give the title compound as a colourless gum, 95mg, 98%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 10 300MHz) δ: 1.41-1.80 (m, 17H), 1.90 (m, 1H), 1.92-2.20 (m, 3H), 2.40 (m, 1H), 2.60 (m, 2H), 3.60 (d, 1H), 3.71 (d, 1H), 5.80 (bs, 1H), 7.15-7.30 (m, 5H); LRMS : m/z 388.1 (MH<sup>+</sup>)

Example 43

- 15 2-[[1-[[1-(5-Methyl-1,3,4-thiadiazol-2-yl)amino]carbonyl]cyclopentyl]methyl]-4-phenylbutanoic acid



- A mixture of the benzyl ester from preparation 70 (187mg, 0.39mmol) and 10% palladium on charcoal (80mg) in ethanol (20ml) was hydrogenated at 60 psi for 18 20 hours. Tlc analysis showed starting material remaining, so additional 10% palladium on charcoal (100mg) was added, and the reaction continued for a further 5 hours. Tlc analysis again showed starting material remaining, so additional catalyst (100mg) was added, and hydrogenation continued for 18 hours. The mixture was filtered

through Arbocel®, and the filtrate concentrated under reduced pressure, and azeotroped with dichloromethane. The crude product was purified by chromatography on silica gel using a Biotage® column, and dichloromethane:methanol (95:5) as eluant to afford the title compound as a clear oil,  
5 80mg, 53%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 1.51-1.89 (m, 9H), 2.03 (m, 1H), 2.20 (m, 1H), 2.40 (m, 2H), 2.60 (m, 5H), 7.15-7.30 (m, 5H); LRMS : m/z 387.8 (MH<sup>+</sup>).

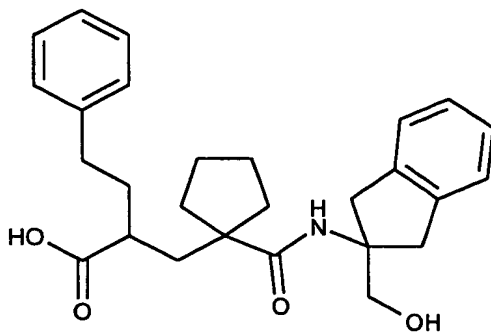
#### Example 44

10 (+)-2-([1-([2-(Hydroxymethyl)-2,3-dihydro-1H-inden-2-yl]amino)carbonyl)cyclopentyl]-methyl)-4-phenylbutanoic acid

and

#### Example 45

15 (-)-2-([1-([2-(Hydroxymethyl)-2,3-dihydro-1H-inden-2-yl]amino)carbonyl)cyclopentyl]-methyl)-4-phenylbutanoic acid



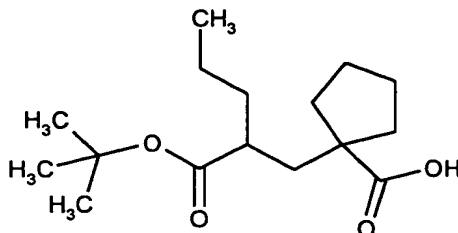
2-([1-([2-(Hydroxymethyl)-2,3-dihydro-1H-inden-2-yl]amino)carbonyl)cyclopentyl]-methyl)-4-phenylbutanoic acid (WO 9110644) may be purified by standard HPLC procedures using an AD column and hexane:isopropanol:trifluoroacetic acid  
20 (70:30:0.2) as eluant, to give the title compound of example 44, 99.5% ee; [ $\alpha$ ]<sub>D</sub> = +9.1 ° (c = 1.76 in ethanol); and the title compound of example 45, 99.5% ee; [ $\alpha$ ]<sub>D</sub> = -10.5° (c = 2.2 in ethanol).

The following Preparations describe the preparation of certain intermediates used in the preceding Examples.

### NEPi Preparations

#### 5 Preparation 1

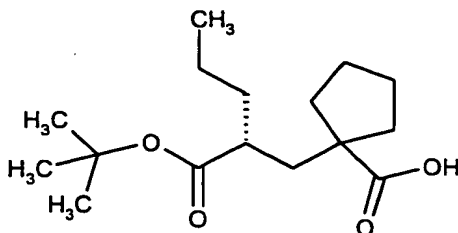
##### 1-[2-(*tert*-Butoxycarbonyl)-4-pentyl]-cyclopentane carboxylic acid



A mixture of 1-[2-(*tert*-butoxycarbonyl)-4-pentenyl]-cyclopentane carboxylic acid (EP 274234) (23g, 81.5mmol) and 10% palladium on charcoal (2g) in dry ethanol (200ml) was hydrogenated at 30psi and room temperature for 18 hours. The reaction mixture was filtered through Arbocel®, and the filtrate evaporated under reduced pressure to give a yellow oil. The crude product was purified by column chromatography on silica gel, using ethyl acetate:pentane (40:60) as the eluant, to provide the desired product as a clear oil, 21g, 91%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 0.86 (t, 3H), 1.22-1.58 (m, 15H), 1.64 (m, 4H), 1.78 (dd, 1H), 2.00-2.18 (m, 3H), 2.24 (m, 1H); LRMS : m/z 283 (M-H)<sup>+</sup>

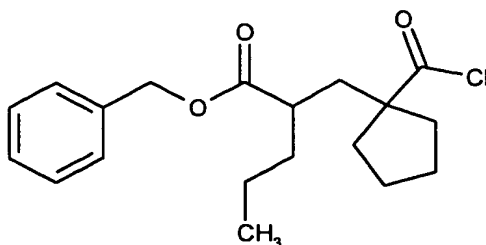
#### Preparation 2

##### 1-[(2*R*)-2-(*tert*-Butoxycarbonyl)-4-pentyl]-cyclopentane carboxylic acid

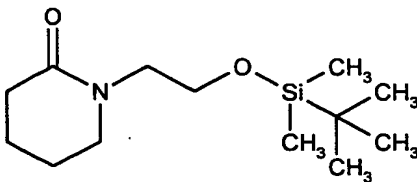


A mixture of (R)-1-[2-(*tert*-butoxycarbonyl)-4-pentenyl]-cyclopentane carboxylic acid (WO 9113054) (10g, 35.4mmol) and 10% palladium on charcoal (600mg) in dry ethanol (25ml) was hydrogenated at 1 atm. and room temperature for 18 hours. The reaction mixture was filtered through Arbocel®, and the filtrate evaporated under reduced pressure to give the title compound as a yellow oil, 9.6g, 95%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 0.86 (t, 3H), 1.22-1.58 (m, 15H), 1.64 (m, 4H), 1.78 (dd, 1H), 2.00-2.18 (m, 3H), 2.24 (m, 1H); [α]<sub>D</sub> = -3.3° (c = 0.09, ethanol).

#### Preparation 3

Benzyl 2-([1-(chlorocarbonyl)cyclopentyl]methyl)pentanoate

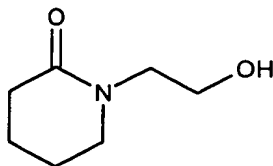
Oxalyl chloride (1.15ml, 13.2mmol) was added to an ice-cooled solution of 1-{2-[(benzyloxy)carbonyl]pentyl}cyclopentanecarboxylic acid (EP 274234) (2.0g, 6.3mmol) in dry dichloromethane (20ml), and the solution stirred at room temperature for 2 hours. The reaction mixture was concentrated under reduced pressure and the residue azeotroped with dichloromethane (3x), to give the title compound as a golden oil, 2.1g; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.88 (t, 3H), 1.28 (m, 2H), 1.43 (m, 2H), 1.63 (m, 6H), 2.00 (m, 1H), 2.08-2.35 (m, 3H), 2.44 (m, 1H), 5.15 (s, 2H), 7.28 (m, 5H).

Preparation 41-(2-{[tert-Butyl(dimethyl)silyl]oxy}ethyl)-2-piperidinone

Sodium hydride (807mg, 60% dispersion in mineral oil, 20.18mmol) was added portionwise to a solution of d-valerolactam (2.0g, 20.2mmol) in tetrahydrofuran (100ml) under nitrogen. (2-Bromoethoxy)(tert-butyl)dimethylsilane (4.33ml, 20.2mmol) was added portionwise, and the reaction heated at 70°C for 18 hours. Water (50ml) was added to the cooled reaction, the mixture concentrated *in vacuo*, to remove the tetrahydrofuran, and extracted with ethyl acetate (200ml). The organic solution was dried (MgSO<sub>4</sub>), and evaporated under reduced pressure to give a yellow oil. The crude product was purified by column chromatography on silica gel using an elution gradient of dichloromethane:methanol (98:2 to 97:3) to give the title compound, 3.25g; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.00 (s, 6H), 0.83 (s, 9H), 1.75 (m, 4H), 2.35 (m, 2H), 3.39 (m, 4H), 3.75 (t, 2H); LRMS : m/z 257.9 (M<sup>+</sup>)

### Preparation 5

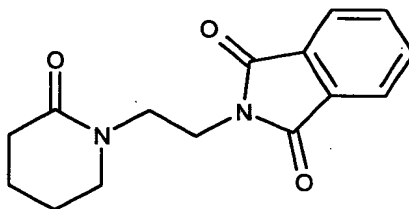
#### 1-(2-Hydroxyethyl)-2-piperidinone



5 Tetra-n-butylammonium fluoride (14ml, 1M solution in tetrahydrofuran, 14mmol) was added to a solution of the lactam from preparation 4 (3.3g, 12.8mmol) in tetrahydrofuran (50ml), and the reaction stirred at room temperature for 2 hours. The mixture was concentrated under reduced pressure, the residue azeotrope with dichloromethane, and purified by column chromatography on silica gel using an elution gradient of dichloromethane:methanol (97:3 to 95:5) to give the title compound as an oil; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.80 (m, 4H), 2.40 (t, 2H), 3.38 (t, 10 2H), 3.42 (t, 1H), 3.56 (t, 2H), 3.80 (t, 2H).

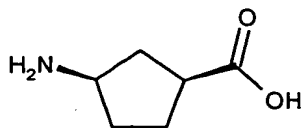
### Preparation 6

#### 2-[2-(2-Oxo-1-piperidinyl)ethyl]-1H-isoindole-1,3(2H)-dione

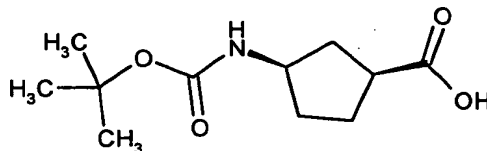


15 Pthalimide (952mg, 6.47mmol) was added to a solution of the alcohol from preparation 5 (842mg, 5.88mmol) in tetrahydrofuran (30ml), and the mixture sonicated until a solution was obtained. Polymer supported triphenyl phosphine (2.5g, 7.5mmol) and diethyl azodicarboxylate (1.15ml, 7.31mmol) were added, and the reaction stirred at room temperature for 18 hours. The mixture was filtered 20 through Arbocel®, the filtrate concentrated under reduced pressure and the residue azeotrope with dichloromethane. The crude product was purified by column chromatography on silica gel using an elution gradient of ethyl acetate:pentane (70:30 to 100:0), to give the title compound as a white foam, 1.6g (containing some 25 impurities); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.60-1.80 (m, 4H), 2.17 (m, 2H), 3.30 (m, 2H), 3.60 (m, 2H), 3.83 (m, 2H), 7.62 (m, 2H), 7.79 (m, 2H); LRMS : m/z 273.2 (MH<sup>+</sup>).



Preparation 7(1S,3R)-3-Aminocyclopentanecarboxylic acid

Platinum oxide (1g) was added to a solution of (1R,4S)-4-amino-cyclopent-2-ene  
5 carboxylic acid (5.3g, 41.7mmol) in water (70ml), and the mixture was hydrogenated  
at 45 psi and room temperature for 18 hours. The mixture was filtered through  
Arbocel®, the filtrate evaporated under reduced pressure, and the residue  
azeotroped with toluene, to afford the title compound as an off-white solid; <sup>1</sup>H NMR  
(D<sub>2</sub>O, 400MHz) δ: 1.70-1.92 (m, 3H), 2.00 (m, 2H), 2.18 (m, 1H), 2.77 (m, 1H), 3.68  
10 (m, 1H); LRMS : m/z 129.8 (MH<sup>+</sup>).

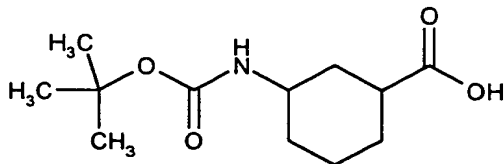
Preparation 8(1S,3R)-3-[(*tert*-Butoxycarbonyl)amino]cyclopentanecarboxylic acid

15 Di-*tert*-butyl dicarbonate (10g, 45.8mmol) was added to an ice-cooled solution of the  
amino acid from preparation 7 (5.4g, 41.8mmol) in dioxan (42.5ml) and sodium  
hydroxide solution (42.5ml, 1N, 42.5mmol), and the reaction stirred at room  
temperature for 18 hours. The reaction mixture was concentrated under reduced  
pressure to remove the dioxan, then acidified to pH 2 using 2N hydrochloric acid. The  
aqueous solution was extracted with ethyl acetate (5x100ml), the combined organic  
20 extracts dried (MgSO<sub>4</sub>) and evaporated under reduced pressure to give a white solid.  
This was triturated with hexane, to give the desired compound as a crystalline solid,  
8.0g, 83%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.41 (s, 9H), 1.58-2.06 (m, 5H), 2.21 (m,  
1H), 2.84 (m, 1H), 4.01 (m, 1H), 4.84 (m, 1H); LRMS: m/z 228 (M-H)<sup>+</sup>.

25

### Preparation 9

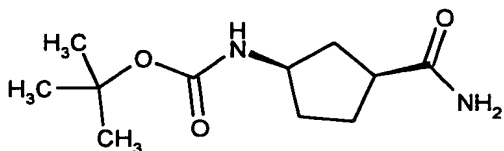
#### 3-[(*tert*-Butoxycarbonyl)amino]cyclohexanecarboxylic acid



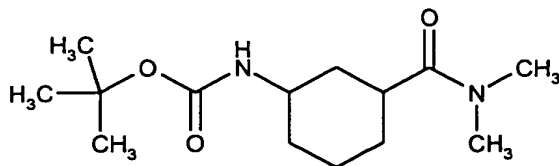
The title compound was obtained as a white solid in 81% yield, from 3-aminocyclohexanecarboxylic acid, following the procedure described in preparation 8; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.04 (m, 1H), 1.19-1.50 (m, 13H), 1.83 (m, 1H), 1.97 (m, 2H), 2.24 (m, 1H), 2.40 (m, 1H), 3.44 (bs, 1H), 4.42 (bs, 1H).

### Preparation 10

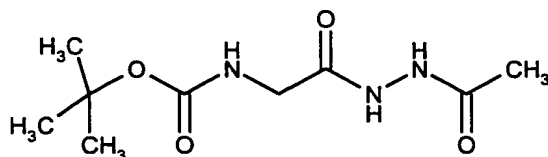
#### *tert*-Butyl (1*R*,3*S*)-3-(aminocarbonyl)cyclopentylcarbamate



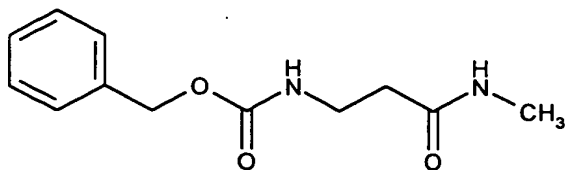
Benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (3.4g, 6.54mmol), 1-hydroxybenzotriazole hydrate (883mg, 6.54mmol), ammonium chloride (467mg, 8.72mmol) and *N*-ethyl-diisopropylamine (3.04ml, 17.5mmol) were added sequentially to a solution of the acid from preparation 8 (1.0g, 4.37mmol) in *N,N*-dimethylformamide (16ml), and the reaction stirred at room temperature for 2 hours. The mixture was diluted with ethyl acetate (100ml), washed with water (3x), and brine, then dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The residual gum was purified by chromatography on silica gel using a Biotage® column, and an elution gradient of dichloromethane:methanol (98:2 to 95:5). The product was triturated with ether to afford the title compound as a white solid, 438mg, 44%; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400MHz) δ: 1.34 (s, 9H), 1.40 (m, 2H), 1.64 (m, 3H), 1.90 (m, 1H), 2.55 (m, 1H), 3.70 (m, 1H), 6.70 (bs, 1H), 6.80 (d, 1H), 7.22 (bs, 1H).

Preparation 11*tert*-Butyl 3-[(dimethylamino)carbonyl]cyclohexylcarbamate

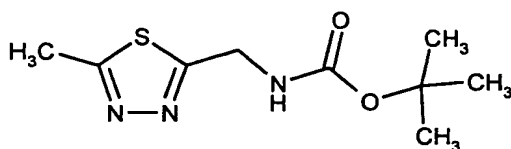
1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.19g, 6.19mmol), 1-  
 5 hydroxybenzotriazole hydrate (840mg, 6.19mmol), N-methylmorpholine (1.1ml,  
 10.1mmol) and finally 33% ethanolic dimethylamine (1.5ml) were added to a solution  
 of the acid from preparation 9 (1.37g, 5.6mmol) in N,N-dimethylformamide (30ml),  
 and the reaction stirred at room temperature for 18 hours. The mixture was  
 concentrated under reduced pressure, the residue diluted with ethyl acetate and  
 10 washed with water (2x). The mixture was dried (MgSO<sub>4</sub>) and evaporated under  
 reduced pressure. The crude product was purified by column chromatography on  
 silica gel using an elution gradient of methanol:dichloromethane (5:95 to 10:90), to  
 give the title compound, 998mg, 66%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 1.12 (m, 1H),  
 1.40 (m, 11H), 1.70 (m, 2H), 1.85 (m, 1H), 2.00 (m, 2H), 2.62 (m, 1H), 2.96 (s, 3H),  
 15 3.05 (s, 3H), 3.50 (m, 1H), 4.50 (m, 1H).

Preparation 12*tert*-Butyl 2-(2-acetylhydrazino)-2-oxoethylcarbamate

20 2-Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (7.06g, 28.5mmol) was added to a  
 solution of N-*tert*-butoxycarbonylglycine (5.0g, 28.6mmol) in dichloromethane (75ml),  
 and the solution stirred for 15 minutes. Acetic hydrazide (2.6g, 35.1mmol) was  
 added, and the reaction stirred at room temperature for 18 hours. The resulting  
 precipitate was filtered off, and dried *in vacuo*, to afford a white crystalline solid,  
 2.42g. The filtrate was concentrated under reduced pressure, diluted with ether, and  
 25 the resulting precipitate filtered and dried *in vacuo*, to afford additional product as a  
 white solid, 4.4g, 67% in total; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.41 (s, 9H), 2.02 (s, 3H),  
 3.87 (d, 2H), 5.22 (bs, 1H), 8.27 (bs, 1H), 8.84 (bs, 1H); LRMS : m/z 249.2 (MNH<sub>4</sub><sup>+</sup>);  
 Anal. Found: C, 46.41; H, 7.36; N, 17.98, C<sub>9</sub>H<sub>17</sub>N<sub>3</sub>O<sub>4</sub> requires C, 46.66; H, 7.41; N,  
 30 18.13%.

Preparation 13Benzyl 3-(methylamino)-3-oxopropylcarbamate

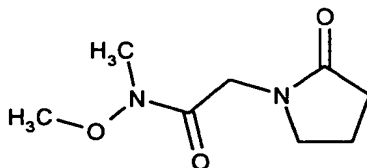
A mixture of N-[(benzyloxy)carbonyl]- $\beta$ -alanine (10g, 44.8mmol), methylamine hydrochloride (3.33g, 49.28mmol), 1-hydroxybenzotriazole hydrate (6.05g, 44.8mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (10.3g, 53.76mmol) and N-methylmorpholine (11.33ml, 103mmol) in dichloromethane (200ml) was stirred at room temperature for 18 hours. The resulting precipitate was filtered off to give the desired product as a colourless foam, and the filtrate evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using an elution gradient of ethyl acetate:hexane (90:10 to 100:0) to give additional product, 7.96g, 75% in total;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300MHz)  $\delta$ : 2.42 (t, 2H), 2.80 (s, 3H), 3.50 (m, 2H), 5.21 (s, 2H), 5.49 (bs, 1H), 5.63 (bs, 1H), 7.36 (m, 5H); Anal. Found: C, 60.68; H, 7.00; N, 11.95.  $\text{C}_{12}\text{H}_{16}\text{N}_2\text{O}_3$  requires C, 61.00; H, 6.83; N, 11.86%.

Preparation 14*tert*-Butyl (5-methyl-1,3,4-thiadiazol-2-yl)methylcarbamate

Lawesson's reagent (960mg, 2.38mmol) was added to a solution of the hydrazide from preparation 12 (500mg, 2.16mmol) in tetrahydrofuran (40ml), and the reaction heated under reflux for 3 hours, then stirred at room temperature for 18 hours. The mixture was evaporated under reduced pressure and the residue purified by column chromatography on silica gel using an elution gradient of ethyl acetate:pentane (70:30 to 80:20) to give an oil. This was dissolved in ethyl acetate (100ml), charcoal (2g) added, the mixture stirred for 10 minutes then filtered. The filtrate was concentrated under reduced pressure, and the residue azeotroped with dichloromethane to afford the title compound as a crystalline solid, 441mg, 89%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz)  $\delta$ : 1.45 (s, 9H), 2.77 (s, 3H), 4.66 (d, 2H), 5.22 (bs, 1H); LRMS :  $m/z$  230.1 ( $\text{MH}^+$ ).

### Preparation 15

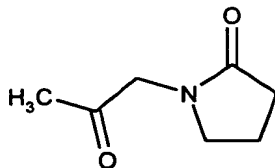
#### N-Methoxy-N-methyl-2-(2-oxo-1-pyrrolidinyl)acetamide



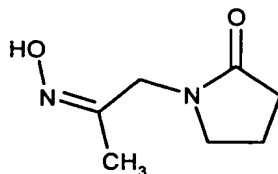
2-Chloro-N-methoxy-N-methylacetamide (3.2g, 23.3mmol) was added to a suspension of 2-pyrrolidinone (2.0g, 23.5mmol) and sodium hydride (940mg, 60% dispersion in mineral oil, 23.5mmol) in tetrahydrofuran (60ml), and the reaction stirred at room temperature for 48 hours. The mixture was quenched with water (150ml), and extracted with ethyl acetate (200ml) and dichloromethane (200ml). The combined organic extracts were dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The residue was triturated with hexane, then ether to afford the title compound as white crystals, 1.8g, 41%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 2.02 (m, 2H), 2.40 (t, 2H), 3.17 (s, 3H), 3.48 (t, 2H), 3.72 (s, 3H), 4.19 (s, 2H); LRMS : m/z 186.9 (MH<sup>+</sup>).

### Preparation 16

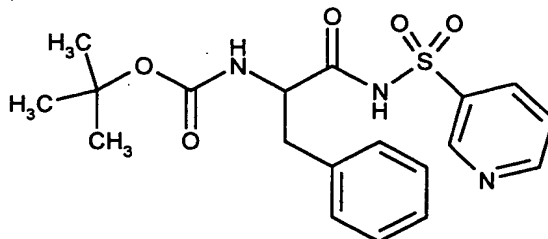
#### 1-(2-Oxopropyl)-2-pyrrolidinone



Methylmagnesium chloride (2.7ml, 3M in tetrahydrofuran, 8.1mmol) was added to a cooled (-20°C) solution of the amide from preparation 15 (1.5g, 8.1mmol) in tetrahydrofuran (50ml), and the reaction allowed to warm to room temperature, then stirred for an hour. The mixture was quenched by the addition of aqueous ammonium chloride solution, then extracted with ethyl acetate (3x50ml). The combined organic solutions were dried (MgSO<sub>4</sub>), and evaporated under reduced pressure to give the title compound as an oil, 645mg, 56%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 2.07 (m, 2H), 2.17 (s, 3H), 2.42 (t, 2H), 3.42 (t, 2H), 4.10 (s, 2H).

Preparation 171-[2-(Hydroxyimino)propyl]-2-pyrrolidinone

Hydroxylamine hydrochloride (316mg, 4.55mmol) and then pyridine (370μl, 4.58mmol) were added to a solution of the amide from preparation 16 (643mg, 4.55mmol) in ethanol (30ml), and the reaction stirred at room temperature for 18 hours. The mixture was evaporated under reduced pressure and the residue purified by column chromatography on silica gel using an elution gradient of dichloromethane:methanol (97:3 to 90:10). The product was triturated with ether to give the title compound as a white solid, 375mg, 53%; <sup>1</sup>Hmr (DMSOd<sub>6</sub>, 400MHz) δ: 1.60 (s, 3H), 1.87 (m, 2H), 2.20 (t, 2H), 3.19 (t, 2H), 3.78 (s, 2H), 10.77 (s, 1H); LRMS : m/z 157.4 (MH<sup>+</sup>).

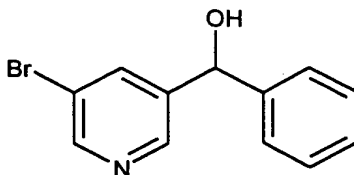
Preparation 1815 *tert*-Butyl 1-benzyl-2-oxo-2-[(3-pyridinylsulfonyl)amino]ethylcarbamate

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (939mg, 4.9mmol), 1-hydroxybenzotriazole hydrate (562mg, 4.15mmol), and N-methylmorpholine (952mg, 9.42mmol) were added to an ice-cold solution of N-*tert*-butoxycarbonyl-L-phenylalanine (1.0g, 3.77mmol) in dichloromethane (20ml), and the mixture stirred for 15 minutes. 3-Pyridinesulphonamide (Mon. für Chemie; 72; 77; 1938) (596mg, 3.77mmol) was added, and the reaction stirred at room temperature for 24 hours. The mixture was evaporated under reduced pressure and the residue partitioned between ethyl acetate (50ml) and water (50ml), and the layers separated. The aqueous layer was extracted well with ethyl acetate, then dichloromethane, the combined organic extracts dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The crude product was purified twice by column chromatography on silica gel, using an elution gradient of ethyl acetate:ethanol (100:0 to 90:10) to give the desired product as a white foam, 1.01g, 66; <sup>1</sup>H NMR (DMSOd<sub>6</sub>, 300MHz) δ: 1.30 (s, 9H), 2.77

(m, 1H), 2.97 (m, 1H), 3.84 (m, 1H), 5.95 (bs, 1H), 6.96 (m, 2H), 7.08 (m, 3H), 7.42 (m, 1H), 8.05 (d, 1H), 8.60 (d, 1H), 8.84 (m, 1H);  $[\alpha]_D = -10^\circ$  (0.1% solution in methanol).

## 5 Preparation 19

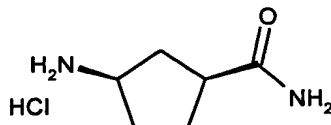
### (5-Bromo-3-pyridinyl)(phenyl)methanol



n-Butyl lithium (17ml, 2.5M in hexanes, 42.5mmol) was added dropwise to cooled (-78°C) solution of 3,5-dibromopyridine (10g, 42.2mmol) in ether (200ml), so as to maintain an internal temperature <-70°C. The mixture was then stirred for 15 minutes, and a solution of benzaldehyde (4.5g, 42.5mmol) in ether (20ml) was added dropwise, again maintaining the temperature <-70°C. The mixture was stirred for 15 minutes, then allowed to warm to room temperature over an hour. The reaction was quenched by the addition of 0.9M ammonium chloride solution (200ml), the layers separated, and the aqueous phase extracted with ether. The combined organic extracts were dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The residual yellow oil was purified by column chromatography on silica gel using an elution gradient of dichloromethane:ether (95:5 to 80:20) to give the title compound as a yellow oil, 7.6g, 68%; <sup>1</sup>H NMR (D<sub>2</sub>O, 300MHz)  $\delta$ : 5.80 (s, 1H), 7.37 (m, 5H), 7.90 (s, 1H), 8.40 (s, 1H), 8.44 (s, 1H).

## Preparation 20

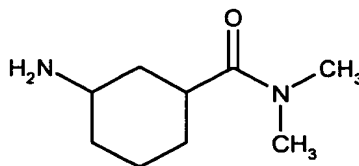
### (1S,3R)-3-Aminocyclopentanecarboxamide hydrochloride



Hydrogen chloride gas was bubbled through an ice-cooled solution of the amide from preparation 10 (438mg, 1.92mmol) in dichloromethane (50ml) for 10 minutes, and the resulting suspension stirred at room temperature for 2 hours. The mixture was purged with nitrogen, then evaporated under reduced pressure. The residue was triturated with ether, to afford the title compound as a solid; <sup>1</sup>H NMR (D<sub>2</sub>O, 400MHz)  $\delta$ : 1.63-1.82 (m, 3H), 1.92-2.07 (m, 2H), 2.19 (m, 1H), 2.82 (m, 1H), 3.62 (m, 1H).

### Preparation 21

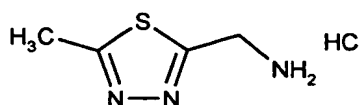
#### 3-Amino-N,N-dimethylcyclohexanecarboxamide



A solution of the amide from preparation 11 (997mg, 3.69mmol) in trifluoroacetic acid (8ml) and dichloromethane (8ml) was stirred at room temperature for 4 hours. The mixture was concentrated under reduced pressure and the residue partitioned between dichloromethane (25ml) and sodium bicarbonate solution (25ml). The pH was adjusted to 9 using sodium hydroxide solution, the layers separated, and the aqueous phase evaporated under reduced pressure. The resulting solid was triturated with hot ethyl acetate, the suspension filtered and the filtrate concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane:methanol:0.88 ammonia (84:14:2) to afford the title compound as a colourless oil, 346mg, 55%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 1.08 (m, 1H), 1.25-1.54 (m, 6H), 1.72 (m, 1H), 1.86 (m, 2H), 2.53-2.75 (m, 2H), 2.96 (s, 3H), 3.03 (s, 3H);

### Preparation 22

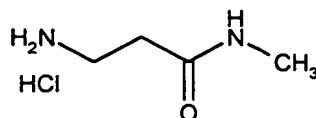
#### (5-Methyl-1,3,4-thiadiazol-2-yl)methylamine hydrochloride



Hydrogen chloride gas was bubbled through an ice-cooled solution of the thiadiazole from preparation 14 (425mg, 1.85mmol) in dichloromethane (50ml) for 15 minutes, and the reaction stirred at room temperature for 1 hour. The mixture was purged with nitrogen, then evaporated under reduced pressure to afford the title compound as a white solid; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 400MHz) δ: 2.75 (s, 3H), 4.48 (m, 2H), 8.80 (bs, 3H).

### Preparation 23

#### 3-Amino-N-methylpropanamide hydrochloride



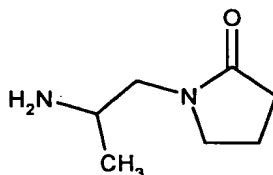
A mixture of the benzyl carbamate from preparation 13 (7.92g, 33.5mmol) and 5% palladium on charcoal (800mg) in ethanol (300ml) was hydrogenated at 50 psi and



room temperature for 4 hours. The reaction mixture was filtered through Arbocel®, washing through with ethanol, and 1N hydrochloric acid (36.9ml, 36.9mmol) was added to the combined filtrate. This solution was evaporated under reduced pressure and the residue azeotrope with dichloromethane to afford the title compound as a colourless foam, 4.66g, <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300MHz) δ: 2.46 (t, 2H), 2.60 (s, 3H), 2.95 (m, 2H), 7.98-8.16 (m, 2H).

#### Preparation 24

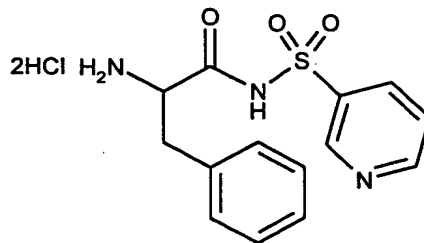
##### 1-(2-Aminopropyl)-2-pyrrolidinone



A mixture of the oxime from preparation 17 (375mg, 2.40mmol) and platinum oxide (300mg) in ethanol (20ml) was hydrogenated at 60psi and room temperature for 18 hours. TLC analysis showed starting material remaining, so additional platinum oxide (100mg) was added and the reaction continued for a further 4 hours. The mixture was filtered through Arbocel®, and the filtrate evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using an elution gradient of dichloromethane:methanol:0.88 ammonia (95:5:0.5 to 90:10:1) to give the title compound as a clear oil, 170mg, 50%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.02 (d, 3H), 1.36 (bs, 2H), 2.00 (m, 2H), 2.38 (t, 2H), 3.00-3.16 (m, 2H), 3.21 (m, 1H), 3.35-3.45 (m, 2H); LRMS : m/z 143 (MH<sup>+</sup>).

#### Preparation 25

##### N-(2-Amino-3-phenylpropanoyl)-3-pyridinesulphonamide dihydrochloride



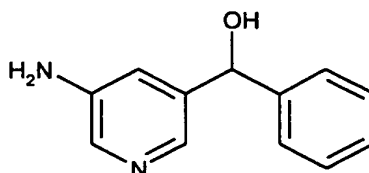
Saturated ethereal hydrochloric acid (40ml) was added to an ice-cold solution of the sulphonamide from preparation 18 (959mg, 2.37mmol) in ethyl acetate (30ml) and ether (10ml), and the solution stirred at room temperature for 18 hours. The reaction mixture was concentrated under reduced pressure and the residue azeotrope with

dichloromethane (3x) to afford the title compound as a white solid, 959mg;  $^1\text{H}$  NMR (DMSO- $d_6$ , 300MHz)  $\delta$ : 3.23-3.50 (m, 1H), 3.70-3.98 (m, 1H), 4.13 (m, 1H), 7.05 (m, 2H), 7.20 (m, 3H), 7.78 (m, 1H), 8.36 (d, 1H), 8.44 (bs, 2H), 8.95 (d, 1H), 9.02 (s, 1H);  $[\alpha]_D = +138^\circ$  (0.5% solution in methanol).

5

#### Preparation 26

##### (5-Amino-3-pyridinyl)(phenyl)methanol

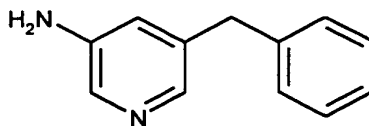


A mixture of the bromide from preparation 19 (2.0g, 7.60mmol) and copper (II) sulphate pentahydrate (350mg, 1.40mmol) in 0.88 ammonia (18ml) was heated at 135°C in a sealed vessel for 24 hours. Sodium hydroxide solution (1N, 10ml) was added to the cooled solution, and the mixture was then extracted with ether (6x). The combined organic extracts were dried (MgSO $_4$ ), and concentrated under reduced pressure to a low volume. The resulting precipitate was filtered, washed with ether and dried to give the title compound as a solid, 1.25g, 83%; mp 92-94°C;  $^1\text{H}$  NMR (DMSO- $d_6$ , 300MHz)  $\delta$ : 5.22 (s, 2H), 5.59 (d, 1H), 5.86 (d, 1H), 6.83 (s, 1H), 7.20 (m, 1H), 7.34 (m, 4H), 7.78 (m, 2H).

15

#### Preparation 27

##### 5-Benzyl-3-pyridinylamine

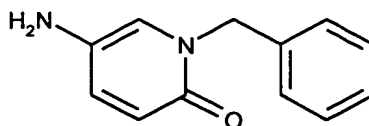


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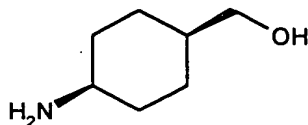
30

A mixture of the alcohol from preparation 26 (700mg, 3.5mmol) and 5% palladium on charcoal (70mg) in hydrochloric acid (5ml, 1N) and ethanol (20ml) was hydrogenated at 30 psi and room temperature for 6 hours. The mixture was filtered through Arbocel®, and the filtrate concentrated under reduced pressure. The residue was basified using aqueous sodium bicarbonate solution, extracted with dichloromethane (3x), and the combined organic extracts dried (MgSO $_4$ ), and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane:methanol:0.88 ammonia (92:8:0.4) as eluant, to give the title compound as a solid, 500mg, 78%; mp 107-109°C;  $^1\text{H}$  NMR (CDCl $_3$ , 300MHz)  $\delta$ : 3.61 (bs, 2H), 3.94 (s, 2H), 6.78 (s, 1H), 7.24 (m, 5H), 7.98 (s, 2H).

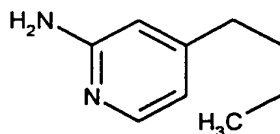
Preparation 285-Amino-1-benzyl-2(1H)-pyridinone

- 5 A mixture of 1-benzyl-5-nitro-1H-pyridin-2-one (Justus Liebigs Ann. Chem. 484; 1930; 52) (1.0g, 4.35mmol), and granulated tin (3.5g, 29.5mmol) in concentrated hydrochloric acid (14ml) was heated at 90°C for 1.5 hours. The cooled solution was diluted with water, neutralised using sodium carbonate solution, and extracted with ethyl acetate (250ml in total). The combined organic extracts were filtered, dried
- 10 (MgSO<sub>4</sub>), and evaporated under reduced pressure to give the title compound as a pale green solid, (turned blue with time), 440mg, 51%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 250MHz) δ: 4.12-4.47 (bs, 2H), 5.00 (s, 2H), 6.31 (d, 1H), 6.86 (s, 1H), 7.07 (m, 1H), 7.14-7.42 (m, 5H).

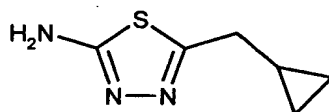
15 Preparation 29

Cis-(4-Aminocyclohexyl)methanol

- Lithium aluminium hydride (14ml, 1M solution in tetrahydrofuran, 14mmol) was added dropwise to an ice-cooled solution of cis-4-aminocyclohexanecarboxylic acid (1.33g, 9.29mmol) in tetrahydrofuran (50ml), and once addition was complete, the reaction
- 20 was heated under reflux for 6 hours. The resulting suspension was cooled to 5°C, and water (0.6ml), aqueous sodium hydroxide solution (1.1ml, 2M), then water (0.6ml) were added sequentially. The resulting suspension was filtered, and the filtrate evaporated under reduced pressure to give an oil, which was used without
- 25 further purification; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 1.40-1.80 (m, 12H), 3.00 (m, 1H), 3.55 (d, 2H); LRMS : m/z 130.2 (MH<sup>+</sup>).

Preparation 302-Amino-4-butylpyridine

A mixture of 4-butylpyridine (5.0g, 37.0mmol) and 95% sodium amide (1.7g, 40.7mmol) in xylene (10ml) was heated at 150°C for 18 hours. The cooled mixture was diluted with ether (100ml) and extracted with 2N hydrochloric acid (twice). The aqueous extracts were basified using sodium hydroxide solution, and re-extracted with ether. These combined organic extracts were dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The residual oil was purified by column chromatography on silica gel using dichloromethane:methanol:0.88 ammonia (97:3:0.15) as eluant, to afford the title compound as a crystalline solid, 2.1g, 38%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.96 (t, 3H), 1.38 (m, 2H), 1.60 (m, 2H), 2.52 (t, 2H), 4.38 (bs, 2H), 6.38 (s, 1H), 6.55 (d, 1H), 7.98 (d, 1H); Anal. Found: C, 72.01; H, 9.47; N, 18.53. C<sub>9</sub>H<sub>14</sub>N<sub>2</sub> requires C, 71.96; H, 9.39; N, 18.65%.

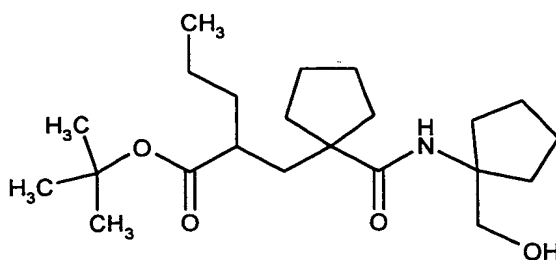
Preparation 315-(Cyclopropylmethyl)-1,3,4-thiadiazol-2-amine

Oxalyl chloride (3.13ml, 35.9mmol) and N,N-dimethylformamide (1 drop) were added to a solution of cyclopropylacetic acid (3g, 29.9mmol) in dichloromethane (30ml), and the reaction stirred at room temperature for 18 hours. The mixture was concentrated under reduced pressure and azeotroped with dichloromethane to give a brown oil.

A mixture of this intermediate acid chloride (887mg, 7.48mmol) and thiosemicarbazide (455mg, 4.99mmol) were heated at 70°C for 18 hours, then cooled. Water was added, the mixture basified to pH 9 using 50% aqueous sodium hydroxide solution, and the resulting precipitate filtered and dried, to give a cream solid, 410mg, 53%; <sup>1</sup>H NMR (CD<sub>3</sub>OD, 400MHz) δ: 0.28 (m, 2H), 0.60 (m, 2H), 1.02 (m, 1H), 2.77 (d, 2H); LRMS : m/z 155.2 (MH<sup>+</sup>).

Preparation 33

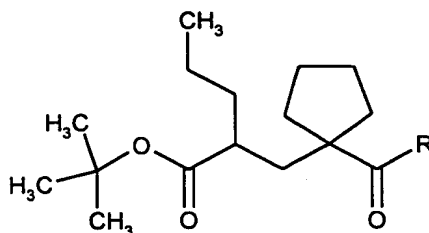
tert-Butyl 2-{{1-({[1-(hydroxymethyl)cyclopentyl]amino}carbonyl)-cyclopentyl]methyl}pentanoate



5 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (41mg, 0.21mmol), 1-hydroxybenzotriazole hydrate (27mg, 0.2mmol), N-methylmorpholine (35μl, 0.31mmol) and finally 1-amino-1-cyclopentanemethanol (25mg, 0.22mmol) were added to a solution of the acid from preparation 1 (150mg, 0.53mmol) in N,N-dimethylformamide (3ml), and the reaction stirred at 90°C for 18 hours. The cooled  
10 solution was diluted with ethyl acetate (90ml), washed with water (3x25ml), and brine (25ml), then dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The crude product was purified by chromatography on silica gel, using ethyl acetate:pentane (30:70) as the eluant to afford the title compound, 38mg, 57%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.88 (t, 3H), 1.29 (m, 3H), 1.41-1.78 (m, 26H), 1.78-1.98 (m, 4H), 2.04  
15 (m, 1H), 2.26 (m, 1H), 3.59 (dd, 1H), 3.70 (dd, 1H), 4.80 (t, 1H), 5.81 (s, 1H); LRMS : m/z 380 (MH<sup>+</sup>).

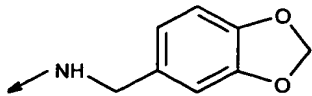
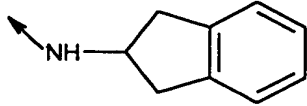
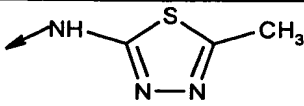
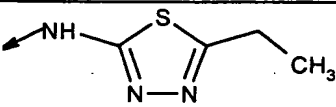
Preparations 34 to 43

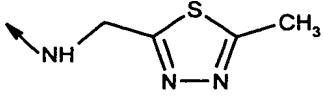
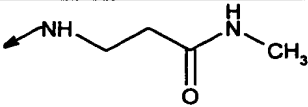
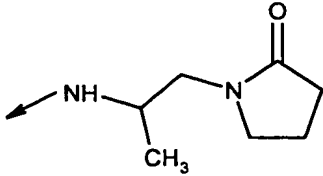
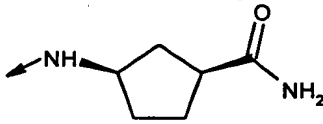
The following compounds of general structure:

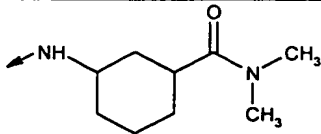
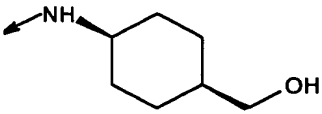


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were prepared from the acid from preparation 1 and the appropriate amine compound, following a similar procedure to that described in preparation 33.

Pre p	R	Starting amine	Yield (%)	Data
34		Piperonylamine	88	$^1\text{H NMR}$ ( $\text{CDCl}_3$ , 400MHz) $\delta$ : 0.85 (t, 3H), 1.26 (m, 4H), 1.42 (s, 9H), 1.46 (m, 2H), 1.59-1.75 (m, 5H), 1.95 (m, 2H), 2.06 (m, 1H), 2.22 (m, 1H), 4.26 (dd, 1H), 4.39 (dd, 1H), 5.95 (m, 3H), 6.78 (m, 3H). LRMS : m/z 418.3 ( $\text{MH}^+$ )
35 <sup>1</sup>		2-Aminoindan hydrochloride	40	$^1\text{H NMR}$ ( $\text{CDCl}_3$ , 400MHz) $\delta$ : 0.87 (t, 3H), 1.25 (m, 3H), 4H), 1.42 (m, 12H), 1.56-1.70 (m, 4H), 1.90 (m, 2H), 2.02 (m, 1H), 2.22 (m, 1H), 2.80 (m, 2H), 3.35 (m, 2H), 4.76 (m, 1H), 5.86 (d, 1H), 7.19 (m, 4H). LRMS : m/z 400.3 ( $\text{MH}^+$ )
36 <sup>2</sup>		2-Amino-5-methyl-1,3,4-thiadiazole	76	$^1\text{H NMR}$ ( $\text{CDCl}_3$ , 400MHz) $\delta$ : 0.82 (t, 3H), 1.20-1.85 (m, 20H), 2.18 (m, 4H), 2.67 (s, 3H), 9.80 (bs, 1H). LRMS : m/z 382.3 ( $\text{MH}^+$ )
37 <sup>2</sup>		2-Amino-5-ethyl-1,3,4-thiadiazole	92	$^1\text{H NMR}$ ( $\text{CDCl}_3$ , 300MHz) $\delta$ : 0.82 (t, 3H), 1.20-1.80 (m, 22H), 1.84 (m, 1H), 2.20 (m, 4H), 3.04 (q, 2H), 9.10 (bs, 1H). LRMS : m/z 396.2 ( $\text{MH}^+$ )

Pre p	R	Starting amine	Yield (%)	Data
38		Preparation 22	77	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 300MHz) δ: 0.84 (t, 3H), 1.20-1.38 (m, 4H), 1.42 (s, 9H), 1.44-1.76 (m, 7H), 1.95-2.12 (m, 3H), 2.20 (m, 1H), 2.76 (s, 3H), 4.74 (dd, 1H), 4.82 (dd, 1H), 6.54 (bs, 1H). LRMS : m/z 396.2 (MH <sup>+</sup> )
39 <sup>1,2</sup>		Preparation 23	60	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 300MHz) δ: 0.88 (t, 3H), 1.21-1.38 (m, 3H), 1.40-1.70 (m, 17H), 1.88-2.04 (m, 3H), 2.20 (m, 1H), 2.39 (t, 2H), 2.80 (d, 3H), 3.53 (m, 2H), 6.13 (bs, 1H), 6.40 (m, 1H). LRMS : m/z 369.5 (MH <sup>+</sup> )
40 <sup>2</sup>		Preparation 24	70	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 300MHz) δ: 0.82 (m, 3H), 1.16 (2xd, 3H), 1.20-1.72 (m, 21H), 1.83 (m, 1H), 1.98 (m, 3H), 2.17 (m, 1H), 2.38 (m, 2H), 1.96 (m, 1H), 3.34 (m, 1H), 3.54-3.62 (m, 2H), 4.15-4.20 (m, 1H), 6.21-6.35 (2xbd, 1H). LRMS : m/z 409.3 (MH <sup>+</sup> ).
41 <sup>2</sup>		Preparation 20	94	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) δ: 0.82 (t, 3H), 1.19-1.38 (m, 4H), 1.42 (m, 12H), 1.60 (m, 3H), 1.74-2.02 (m, 10H), 2.18 (m, 1H), 2.78 (m, 1H), 4.38 (m, 1H), 5.32 (bs, 1H), 5.57 (bs, 1H), 7.28 (bs, 1H). LRMS : m/z 395 (MH <sup>+</sup> )

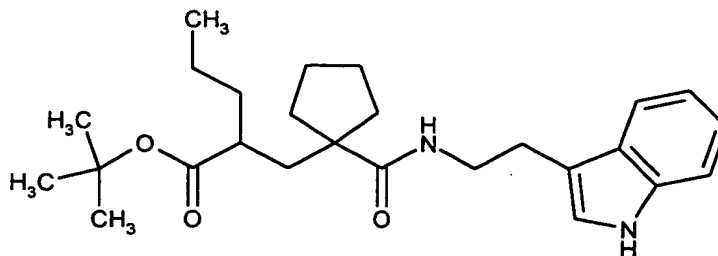
Pre p	R	Starting amine	Yield (%)	Data
42 <sup>2</sup>		Preparation 21	91	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 300MHz) δ: 0.86 (t, 3H), 1.18-1.78 (m, 25H), 1.84-2.03 (m, 6H), 2.22 (m, 1H), 2.68 (m, 1H), 2.96 (s, 3H), 3.03 (s, 3H), 3.84 (m, 1H), 5.78 (m, 1H). LRMS : m/z 437.7 (MH <sup>+</sup> )
43 <sup>2</sup>		Preparation 29	99	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 300MHz) δ: 0.85 (t, 3H), 1.20-1.79 (m, 30H), 1.90 (m, 2H), 2.05 (m, 1H), 2.24 (m, 1H), 3.56 (m, 2H), 4.04 (m, 1H), 5.82 (bd, 1H). LRMS : m/z 396.4 (MH <sup>+</sup> )

1 = reaction conducted at room temperature

2 = Methanol:dichloromethane was used as the column eluant

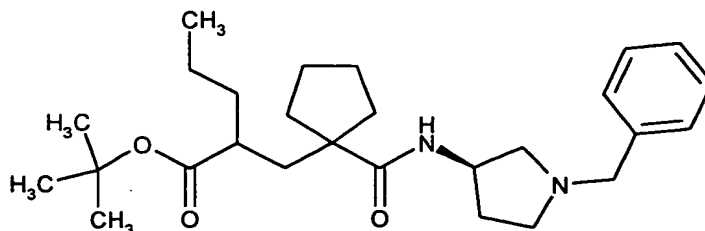
#### Preparation 44

5 tert-Butyl 2-([1-([2-(1H-indol-3-yl)ethyl]amino)carbonyl]cyclopentyl)methyl]pentanoate

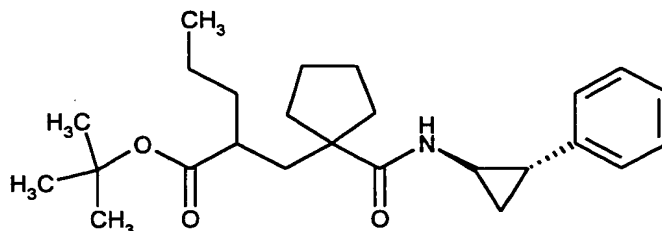


10 The title compound was obtained as a pale yellow oil in 80% yield from the acid from preparation 1 and tryptamine, following a similar procedure to that described in preparation 33, except the reaction was performed in dichloromethane at room temperature; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.86 (t, 3H), 1.26 (m, 3H), 1.42 (m, 11H), 1.50-1.69 (m, 6H), 1.83 (m, 1H), 1.90-2.05 (m, 2H), 2.22 (m, 1H), 2.99 (t, 3H), 3.60 (m, 2H), 5.78 (m, 1H), 7.06 (s, 1H), 7.14 (m, 1H), 7.20 (m, 1H), 7.38 (d, 1H), 7.63 (d, 1H), 8.02 (bs, 1H); LRMS : m/z 427.5 (MH<sup>+</sup>).



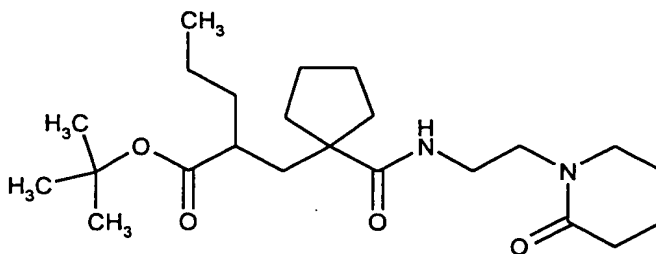
**Preparation 45***tert*-Butyl 2-[(1-[(3*S*)-1-benzylpyrrolidinyl]amino)cyclopentyl)methyl]pentanoate

The title compound was obtained quantitatively from the acid from preparation 1 and (3*S*)-1-benzyl-3-aminopyrrolidine, following a similar procedure to that described in preparation 44; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.84 (t, 3H), 1.10-1.76 (m, 21H), 1.90-2.05 (m, 3H), 2.20-2.38 (m, 3H), 2.58 (m, 2H), 2.84 (m, 1H), 3.62 (s, 2H), 4.45 (m, 1H), 6.02 (m, 1H), 7.33 (m, 5H).

**Preparation 46***tert*-Butyl 2-[1-[(*cis*-2-phenylcyclopropyl)amino]carbonyl]-cyclopentyl)methyl]pentanoate

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (81mg, 0.42mmol), *N*-methylmorpholine (0.15ml, 1.06mmol) and tranylcypromine hydrochloride (60mg, 0.35mmol) were added to a solution of the acid from preparation 1 (100mg, 0.35mmol) in dichloromethane (10ml), and the reaction stirred at room temperature for 18 hours. The reaction mixture was evaporated under reduced pressure and the residue purified by column chromatography on silica gel using an elution gradient of dichloromethane:methanol (98:2 to 95:5) to afford the title compound as a yellow oil, 85mg, 55%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.88 (t, 3H), 1.16 (m, 1H), 1.20-1.58 (m, 16H), 1.63 (m, 5H), 1.90-2.14 (m, 4H), 2.23 (m, 1H), 2.90 (m, 1H), 6.00 (m, 1H), 7.19 (m, 3H), 7.24 (m, 2H); LRMS : *m/z* 400 (MH<sup>+</sup>).

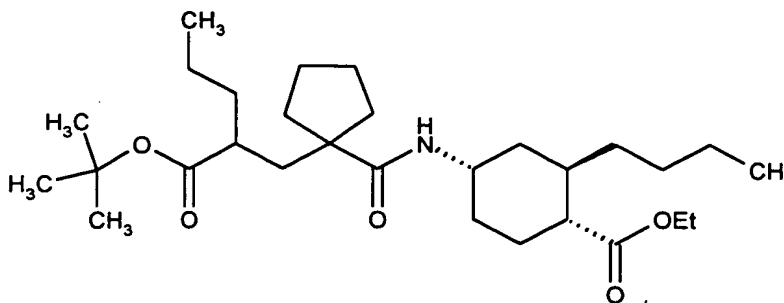
**Preparation 47***tert*-Butyl 2-[1-[(2-(2-oxo-1-piperidinyl)ethyl)amino]carbonyl]cyclopentyl)methyl]pentanoate



Hydrazine monohydrate (34μl, 0.70mmol) was added to a solution of the compound from preparation 6 (171mg, 0.63mmol) in ethanol (10ml), and the reaction heated under reflux for 5 hours. The cooled mixture was filtered, the filtrate concentrated under reduced pressure, the residue suspended in dichloromethane, and the suspension re-filtered. The resulting filtrate was concentrated under reduced pressure, and the residue purified by column chromatography on silica gel using dichloromethane:methanol:0.88 ammonia (90:10:1) as eluant to give the amine, 16mg. The acid from preparation 1 (32mg, 0.11mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (25mg, 0.13mmol), 1-hydroxybenzotriazole hydrate (17mg, 0.13mmol), and N-methylmorpholine (25μl, 0.23mmol) were added to a solution of this amine in N,N-dimethylformamide (2ml), and the reaction stirred at room temperature for 18 hours. The mixture was partitioned between ethyl acetate and water, and the layers separated. The organic phase was washed with water (2x), dried (MgSO<sub>4</sub>), and evaporated under reduced pressure. The residual oil was purified by column chromatography on silica gel using an elution gradient of dichloromethane:methanol (98.5:1.5 to 95:5) to afford the title compound as an oil, 43mg, 17%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.82 (t, 3H), 1.22 (m, 3H), 1.38-1.65 (m, 17H), 1.58 (m, 4H), 1.95 (m, 3H), 2.17 (m, 1H), 2.37 (m, 2H), 3.30 (m, 2H), 3.38 (m, 2H), 3.50 (m, 2H), 6.76 (m, 1H); LRMS : m/z 409.2 (MH<sup>+</sup>)

#### Preparation 48

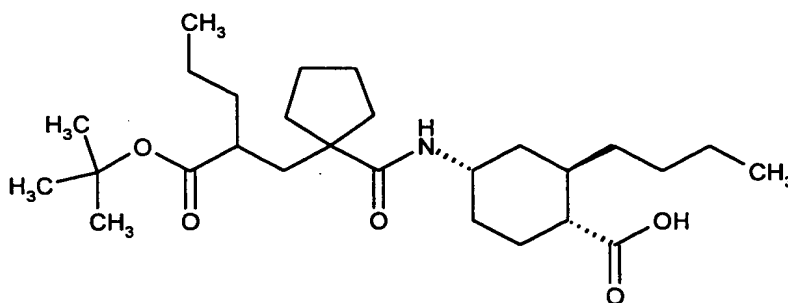
Ethyl (1R,2R,4S)-4-[(1-[2-(tert-butoxycarbonyl)pentyl]cyclopentyl)carbonyl]amino]-2-butylcyclohexanecarboxylate



A mixture of the acid from preparation 1 (109mg, 0.38mmol), (1*R*,2*R*,4*S*)-4-amino-2-butyl-cyclohexanecarboxylic acid ethyl ester hydrochloride (WO, 9009374), (101mg, 0.38mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (95mg, 0.50mmol), 1-hydroxybenzotriazole hydrate (60mg, 0.40mmol) and triethylamine (0.12ml, 0.87mmol) in dichloromethane (3ml), was stirred at room temperature for 16 hours. The mixture was evaporated under reduced pressure, the residue treated with sodium bicarbonate solution and extracted with ethyl acetate. The combined organic extracts were dried (MgSO<sub>4</sub>) and evaporated under reduced pressure to give a gum. The crude product was purified by column chromatography on silica gel using ethyl acetate:pentane (50:50) as eluant, and azeotroped with dichloromethane to afford the title compound, 190mg; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.88 (m, 6H), 1.20-1.40 (m, 13H), 1.40-2.10 (m, 25H), 2.16-2.30 (m, 2H), 4.18 (m, 3H), 5.83 (d, 1H).

#### Preparation 49

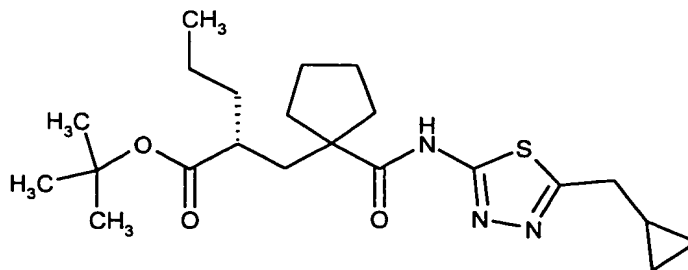
(1*R*, 2*R*,4*S*)-4-[(1-[2-(*tert*-Butoxycarbonyl)pentyl]cyclopentyl)carbonyl]amino]-2-butylcyclohexanecarboxylic acid



A mixture of the ethyl ester from preparation 48 (190mg, 0.39mmol) and 1N sodium hydroxide solution (0.85ml, 0.85mmol) in methanol (1.5ml) was stirred at room temperature for 22 hours. The reaction mixture was acidified to pH 1 using hydrochloric acid (2N), then partitioned between ethyl acetate and water. The layers were separated, and the organic phase was dried (MgSO<sub>4</sub>) and evaporated under reduced pressure to afford the title compound, 130mg, 72%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.86 (m, 6H), 1.20-2.12 (m, 36H), 2.24 (m, 2H), 4.18 (m, 1H), 5.82 (d, 1H); LRMS : m/z 464 (M-H)<sup>-</sup>

#### Preparation 50

*tert*-Butyl (2*R*)-2-[1-[(5-(cyclopropylmethyl)-1,3,4-thiadiazol-2-yl)amino]carbonyl]cyclopentyl[methyl]pentanoate

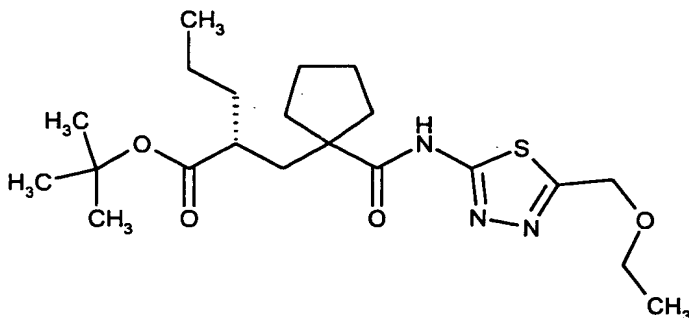


The title compound was prepared from the acid from preparation 2 and the amine from preparation 31, in 65% yield, following the procedure described in preparation 33; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.35 (m, 2H), 0.63 (m, 2H), 0.80 (m, 3H), 1.10 (m, 1H), 1.20-1.94 (m, 20H), 2.19 (m, 4H), 2.93 (t, 2H), 3.50 (s, 1H); LRMS : m/z 422.4 (MH<sup>+</sup>)

[α]<sub>D</sub> = -14.15° (c = 0.082, methanol).

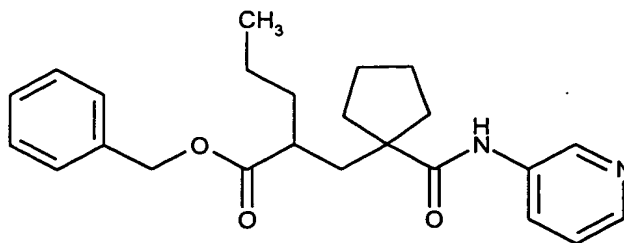
#### Preparation 51

tert-Butyl (2R)-2-([1-([5-(ethoxymethyl)-1,3,4-thiadiazol-2-yl]amino)carbonyl]-cyclopentyl)methyl}pentanoate



The title compound was prepared from the acid from preparation 2 and 5-(ethoxymethyl)-1,3,4-thiadiazol-2-amine, in 51% yield, following the procedure described in preparation 33; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.10-1.78 (m, 25H), 1.82 (m, 1H), 2.19 (m, 5H), 3.48 (s, 1H), 4.82 (s, 2H), 10.16 (brs, 1H); LRMS : m/z 426.4 (MH<sup>+</sup>); [α]<sub>D</sub> = -12.50° (c = 0.08, methanol).

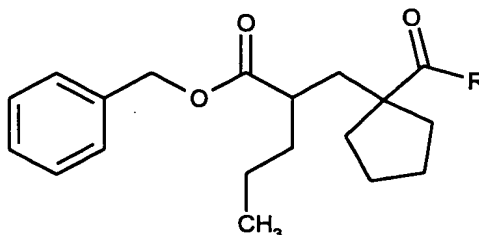
#### Preparation 52

Benzyl 2-({1-[(3-pyridinylamino)carbonyl]cyclopentyl}methyl)pentanoate

Triethylamine (0.11ml, 0.78mmol) was added to a mixture of the acid chloride from preparation 3 (200mg, 0.60mmol) and 2-aminopyridine (61mg, 0.65mmol) in dichloromethane (3ml), and the reaction stirred at room temperature for 16 hours. The mixture was evaporated under reduced pressure, the residue partitioned between sodium bicarbonate solution (5ml) and ethyl acetate (20ml), and the layers separated. The organic phase was dried (MgSO<sub>4</sub>), and evaporated under reduced pressure to give a gum. The crude product was purified by column chromatography on silica gel using ethyl acetate as eluant, to afford the title compound, 130mg; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.82 (t, 3H), 1.21 (m, 3H), 1.40 (m, 1H), 1.43-1.72 (m, 6H), 1.81 (d, 1H), 1.98 (m, 1H), 2.18 (m, 1H), 2.24 (m, 1H), 2.46 (m, 1H), 4.98 (m, 2H), 7.20-7.38 (m, 6H), 7.42 (s, 1H), 8.06 (d, 1H), 8.35 (d, 1H), 8.56 (s, 1H).

15 Preparations 53 to 56

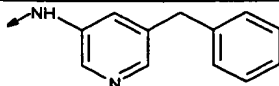
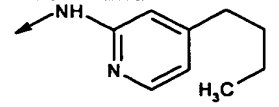
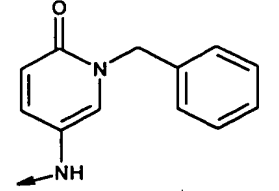
The following compounds of general formula:



were prepared from the acid chloride from preparation 3 and the appropriate amine, following a similar procedure to that described in preparation 52.

20

Prep	R	Yield (%)	Data
53 <sup>1</sup>		90	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 300MHz) δ: 0.84 (t, 3H), 1.24 (m, 2H), 1.40-1.76 (m, 7H), 1.84 (dd, 1H), 1.98 (m, 1H), 2.19 (dd, 1H), 2.28 (m, 1H), 2.56 (m, 1H), 3.98 (s, 2H), 4.99 (dd, 2H), 6.98 (d, 1H), 7.19-7.42 (m, 15H).

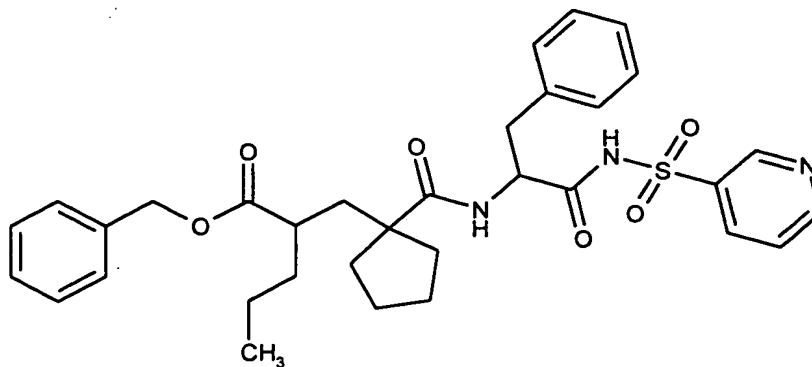
Prep	R	Yield (%)	Data
54		65	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 300MHz) δ: 0.85 (t, 3H), 1.24 (m, 3H), 1.39-1.78 (m, 6H), 1.82 (dd, 1H), 1.98 (m, 2H), 2.20 (dd, 1H), 2.25 (m, 1H), 2.50 (m, 1H), 3.98 (s, 2H), 4.98 (dd, 2H), 7.18-7.40 (m, 10H), 7.45 (s, 1H), 7.98 (s, 1H), 8.23 (s, 1H), 8.42 (s, 1H).
55		30	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) δ: 0.80 (t, 3H), 0.92 (t, 3H), 1.21 (m, 2H), 1.30-1.70 (m, 12H), 1.82 (dd, 1H), 2.04 (m, 1H), 2.20 (m, 2H), 2.50 (m, 1H), 2.58 (t, 2H), 4.98 (dd, 2H), 6.83 (d, 1H), 7.30 (m, 5H), 7.90 (s, 1H), 8.08 (s, 1H), 8.15 (d, 1H).
56 <sup>2</sup>		53	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 300MHz) δ: 0.84 (t, 3H), 1.25 (m, 2H), 1.27-1.99 (m, 10H), 2.07-2.30 (m, 2H), 2.47 (m, 1H), 4.99 (s, 2H), 5.10 (dd, 2H), 6.59 (d, 1H), 7.15 (d, 1H), 7.34 (m, 11H), 8.10 (s, 1H).

1 = dichloromethane used as the column eluant

2 = N-methylmorpholine was used as the base

### Preparation 57

- 5 Benzyl 2-({1-[(1-benzyl-2-oxo-2-[(3-pyridinylsulfonyl)amino]ethyl)amino]-carbonyl}cyclopentyl)methyl)pentanoate

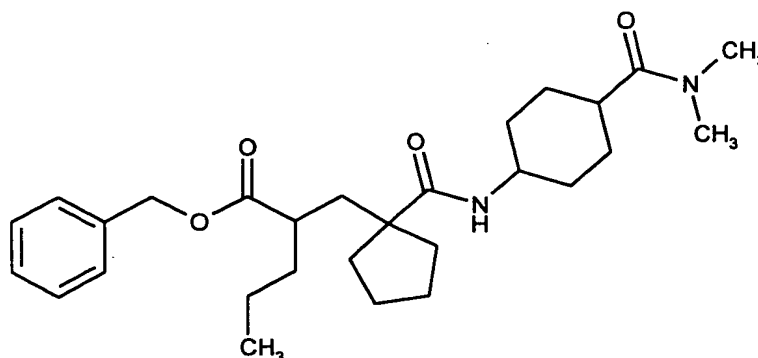


- 10 The amine hydrochloride from preparation 25 (828mg, 2.19mmol) and N-methylmorpholine (2.21g, 21.9mmol) was added to an ice-cold solution of the acid chloride from preparation 3 (737mg, 2.19mmol) in dichloromethane (50ml), and the

reaction stirred at room temperature for 24 hours. The reaction mixture was evaporated under reduced pressure, the residue partitioned between ethyl acetate (50ml) and water (50ml), and the layers separated. The organic phase was washed with brine (25ml), dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using an elution gradient of ethyl acetate:methanol (100:0 to 95:5) to give the title compound as a cream foam, 975mg, 73%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.72 (m, 3H), 0.94-2.20 (m, 17H), 2.84 (m, 1H), 3.00 (m, 1H), 4.18 (m, 1H), 5.00 (m, 2H), 6.95 (m, 2H), 7.02 (m, 3H), 7.38 (m, 6H), 8.06 (m, 1H), 8.60 (m, 1H), 8.87 (s, 1H).

### Preparation 58

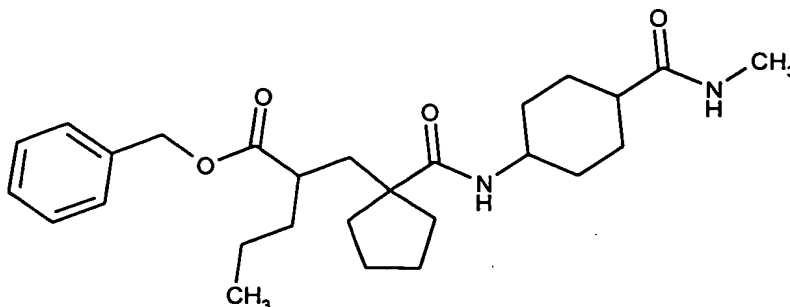
Cis-Benzyl 2-({1-[[4-[(dimethylamino)carbonyl]cyclohexyl]amino]carbonyl}-cyclopentyl)methyl}pentanoate



A mixture of cis-4-[[1-[[2-[(benzyloxy)carbonyl]pentyl]cyclopentyl]carbonyl]amino]-cyclohexanecarboxylic acid (EP 274234) (200mg, 0.45mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (112mg, 0.58mmol), 1-hydroxybenzotriazole hydrate (70mg, 0.46mmol) and dimethylamine (0.56ml, 2M in tetrahydrofuran, 1.12mmol) in dichloromethane (5ml) was stirred at room temperature for 16 hours. The mixture was concentrated under reduced pressure and the residue partitioned between sodium bicarbonate solution and ethyl acetate, and the layers separated. The organic phase was dried (MgSO<sub>4</sub>) and evaporated under reduced pressure to give a gum. The crude product was purified by column chromatography on silica gel using ethyl acetate as eluant to afford the title compound, 150mg; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.82 (t, 3H), 1.22 (m, 3H), 1.32-1.88 (m, H), 2.00 (m, 4H), 2.40 (m, 1H), 2.60 (m, 1H), 2.97 (s, 3H), 3.04 (s, 3H), 4.04 (m, 1H), 5.12 (s, 2H), 5.80 (bd, 1H), 7.37 (m, 5H).

### Preparation 59

Cis-Benzyl 2-({1-[(4-[(methylamino)carbonyl]cyclohexyl)amino]carbonyl}-cyclopentyl)methyl}pentanoate

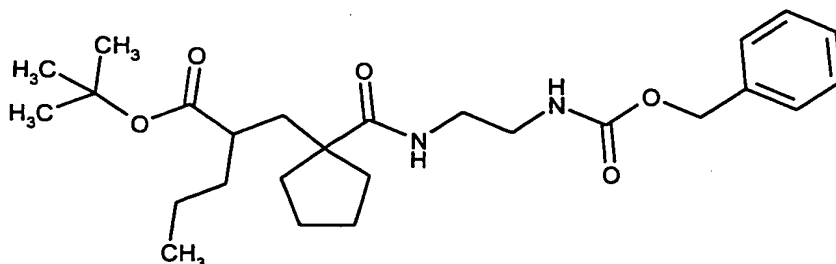


The title compound was prepared in 49% yield from cis-4-[[1-2-  
 5 [(benzyloxy)carbonyl]pentyl]cyclopentyl]carbonyl]amino}cyclohexanecarboxylic acid  
 (EP 274234) and methylamine (2M in tetrahydrofuran), following the procedure  
 described in preparation 58; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.82 (t, 3H), 1.17-2.12 (m,  
 22H), 2.21 (m, 1H), 2.41 (m, 1H), 2.80 (d, 3H), 4.00 (m, 1H), 5.12 (s, 2H), 5.61 (m,  
 1H), 5.79 (d, 1H), 7.38 (m, 5H).

10

Preparation 60

tert-Butyl 2-[(1-[(2-[(benzyloxy)carbonyl]amino)ethyl]amino]carbonyl}-cyclopentyl)methyl]pentanoate



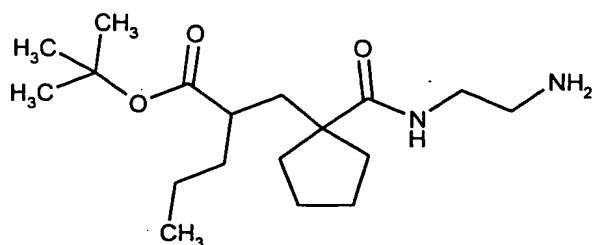
15 The title compound was obtained as a yellow oil in 55% yield, from the acid from  
 preparation 1 and N-benzyloxycarbonyl-1,2-diaminoethane, following a similar  
 procedure to that described in preparation 44; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.84 (t,  
 3H), 1.20-1.38 (m, 3H), 1.40-1.74 (m, 17H), 1.90 (m, 2H), 2.04 (m, 1H), 2.20 (m, 1H),  
 3.32 (m, 3H), 3.44 (m, 1H), 5.10 (s, 2H), 5.61 (m, 1H), 6.20 (m, 1H), 7.36 (m, 5H).

20

Preparation 61

tert-Butyl 2-[(1-[(2-aminoethyl)amino]carbonyl}cyclopentyl)methyl]pentanoate

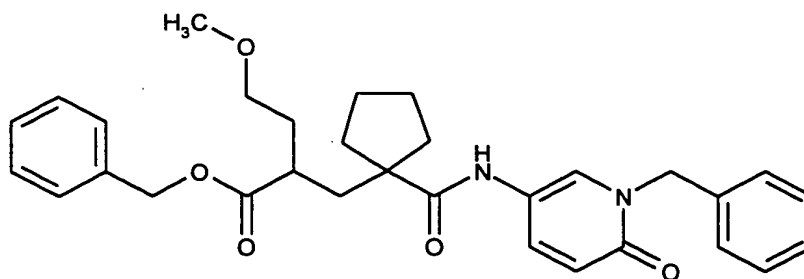




A mixture of the carbamate from preparation 60 (1.43g, 3.10mmol) and 10% palladium on charcoal (200mg) in ethanol (8ml) was hydrogenated at room temperature and 1 atm for 18 hours. The reaction mixture was filtered through Arbocel®, and the filtrate evaporated under reduced pressure to afford the title compound, 920mg, 92%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 0.84 (t, 3H), 1.20-1.38 (m, 3H), 1.40-1.54 (m, 12H), 1.61 (m, 5H), 1.92-2.12 (m, 3H), 2.20 (m, 1H), 2.98 (m, 2H), 3.38 (m, 1H), 3.42 (m, 1H), 3.97 (m, 2H), 6.65 (m, 1H); LRMS : m/z 326.8 (M<sup>+</sup>).

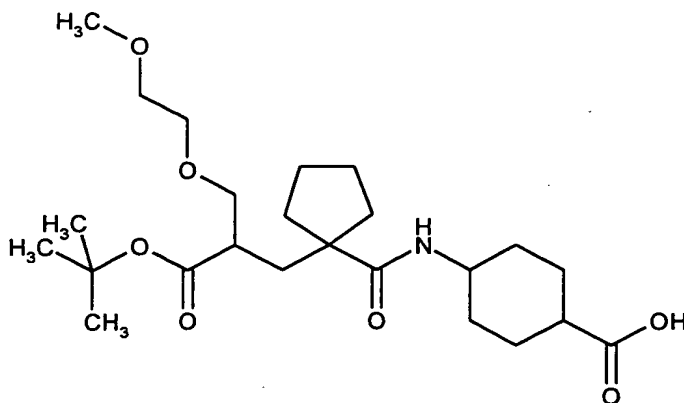
#### 10 Preparation 62

Benzyl 2-[(1-[(1-benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl)cyclopentyl]-methyl]-4-methoxybutanoate

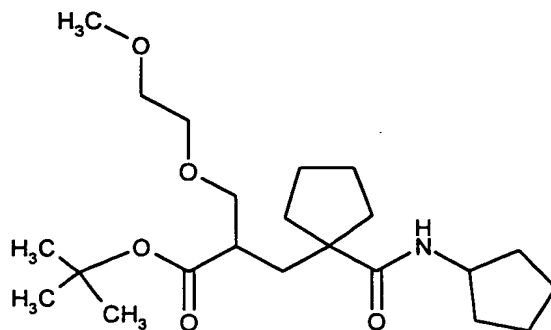


Oxalyl chloride (0.26ml, 3.0mmol) was added to an ice-cooled solution of 1-{2-[(benzyloxy)carbonyl]-4-methoxybutyl}cyclopentanecarboxylic acid (EP 274234) (1.0g, 3.0mmol) and N,N-dimethylformamide (2 drops) in dichloromethane (20ml), and the reaction stirred at room temperature for 2 hours. The solution was concentrated under reduced pressure and the residue azeotroped with dichloromethane (3x10ml). The product was dissolved in dichloromethane (20ml), then cooled in an ice-bath. The amine from preparation 28 (600mg, 3mmol) and N-methylmorpholine (0.6ml, 5.45mmol) were added and the reaction stirred at room temperature for 18 hours. The reaction mixture was concentrated under reduced pressure, and partitioned between water and ether. The organic layer was washed with hydrochloric acid (2N), sodium bicarbonate solution, then water, dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The residual green solid was purified by medium pressure column chromatography on silica gel using ethyl acetate:hexane (90:10) as eluant to afford the title compound, 880mg, 57%; <sup>1</sup>H NMR (CDCl<sub>3</sub>,

300MHz)  $\delta$ : 1.37-2.28 (m, 12H), 2.46-2.64 (m, 1H), 3.20 (s, 3H), 3.31 (m, 2H), 4.97 (dd, 2H), 5.08 (dd, 2H), 6.57 (d, 1H), 7.12 (m, 1H), 7.18-7.48 (m, 10H), 8.08 (d, 1H).

Preparation 634-[[[1-(3-*tert*-Butoxy-2-[(2-methoxyethoxy)methyl]-3-oxopropyl)cyclopentyl]carbonyl]amino]cyclohexanecarboxylic acid

- 5 A mixture of benzyl 4-[[[1-(3-*tert*-butoxy-2-[(2-methoxyethoxy)methyl]-3-oxopropyl)cyclopentyl]carbonyl]amino]cyclohexanecarboxylate (EP 274234), and 10% palladium on charcoal (250mg) in water (10ml) and ethanol (50ml) was hydrogenated at 50 psi and room temperature for 18 hours. The reaction mixture was filtered through Solkaflor®, the filtrate concentrated under reduced pressure and the residue azeotroped with toluene (3x) and then dichloromethane (3x), to give the title compound, 2.0g, 96%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 1.48 (s, 9H), 1.53-1.84 (m, 14H), 1.94-2.10 (m, 5H), 2.60 (m, 2H), 3.40 (s, 3H), 3.41-3.63 (m, 5H), 3.96 (m, 1H), 5.90 (bd, 1H).

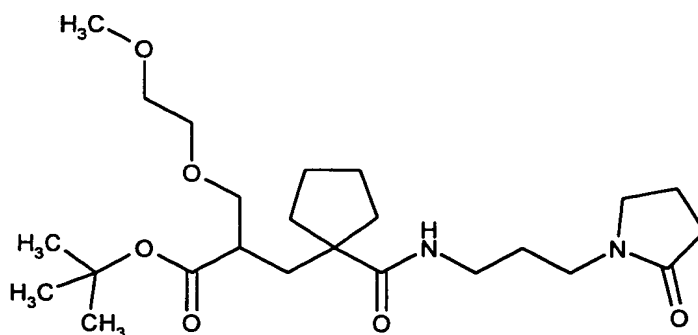
15 Preparation 64*tert*-Butyl 3-[1-[(cyclopentylamino)carbonyl]cyclopentyl]-2-[(2-methoxyethoxy)methyl]propanoate

- 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (197mg, 1.07mmol), 1-hydroxybenzotriazole hydrate (139mg, 1.07mmol), N-methylmorpholine (0.18ml, 1.64mmol) and cyclopentylamine (101μl, 1.07mmol) were added to a solution of 1-(3-*tert*-butoxy-2-[(2-methoxyethoxy)methyl]-3-oxopropyl)-cyclopentanecarboxylic acid

(EP 274234) (400mg, 1.07mmol) in dichloromethane (5ml), and the reaction stirred at room temperature for 22 hours. The reaction was quenched by the addition of water, extracted with dichloromethane (3x), and the combined organic extracts dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using ethyl acetate:pentane (30:70) as eluant to afford the title compound as a clear oil, 320mg, 78%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.22-2.02 (m, 27H), 2.58 (m, 1H), 3.36 (s, 3H), 3.40 (m, 1H), 3.46 (m, 2H), 3.57 (m, 3H), 4.10-4.20 (m, 1H), 5.80 (bs, 1H).

#### 10 Preparation 65

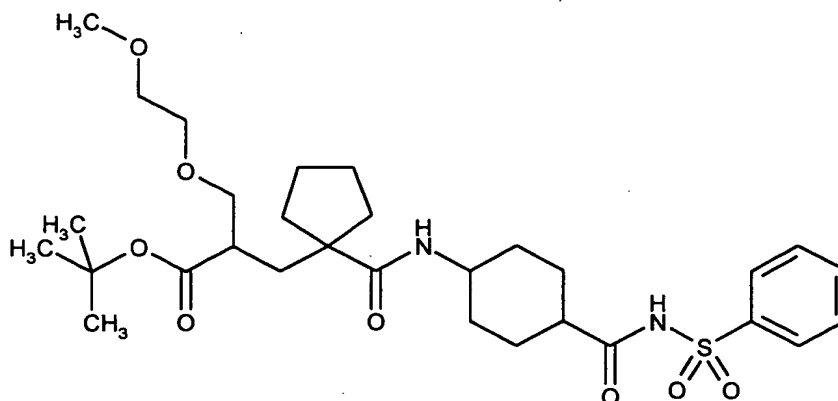
tert-Butyl 3-(2-methoxyethoxy)-2-([1-([3-(2-oxo-1-pyrrolidinyl)propyl]amino)carbonyl]cyclopentyl)methyl]propanoate



The title compound was obtained as a clear oil in 97% yield from 1-{3-*tert*-butoxy-2-[(2-methoxyethoxy)methyl]-3-oxopropyl}-cyclopentanecarboxylic acid (EP 274234) and 1-(3-aminopropyl)-2-pyrrolidinone, following a similar procedure to that described in preparation 64, except dichloromethane:methanol (95:5) was used as the column eluant, <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.41 (s, 9H), 1.50 (m, 2H), 1.60-1.70 (m, 7H), 1.78 (m, 1H), 1.90 (m, 1H), 2.20 (m, 4H), 2.40 (m, 2H), 2.58 (m, 1H), 3.14 (m, 1H), 3.20 (m, 1H), 3.38 (m, 6H), 3.42-3.60 (m, 6H), 7.00 (m, 1H).

Preparation 66

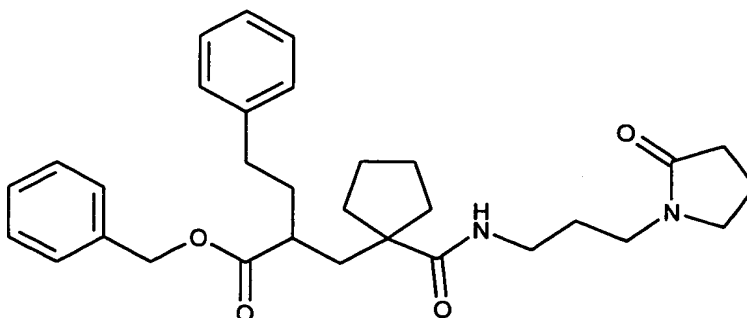
Cis-tert-Butyl 3-(2-methoxyethoxy)-2-[(1-[(4-[(phenylsulfonyl)amino]carbonyl)-cyclohexyl)amino]carbonyl)cyclopentyl)methyl]propanoate



- 5 N,N'-Dicyclohexylcarbodiimide (199mg, 0.97mmol), 4-dimethylaminopyridine (118mg, 0.97mmol) and benzenesulphonamide (152mg, 0.97mmol) were added to an ice-cooled solution of the acid from preparation 63 (400mg, 0.878mmol) in dichloromethane (12ml) and N,N-dimethylformamide (0.5ml), and the reaction stirred at room temperature for 20 hours. The mixture was concentrated under reduced
- 10 pressure and the residue suspended in cold ethyl acetate. The resulting insoluble material was filtered off, the filtrate washed with hydrochloric acid (1N), and water, then dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using an elution gradient of dichloromethane:methanol (95:5 to 90:10) to afford the title compound as a white
- 15 foam, 480mg, 92%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.44 (s, 9H), 1.63 (m, 13H), 1.80 (m, 2H), 1.88 (m, 1H), 1.98 (m, 2H), 2.36 (m, 1H), 2.57 (m, 1H), 3.38 (s, 3H), 3.40 (m, 1H), 3.51 (t, 2H), 3.58 (m, 3H), 3.95 (m, 1H), 5.92 (d, 1H), 7.56 (m, 2H), 7.62 (m, 1H), 8.05 (d, 2H), 8.75 (bs, 1H); LRMS : m/z 618 (MNa<sup>+</sup>).

Preparation 67

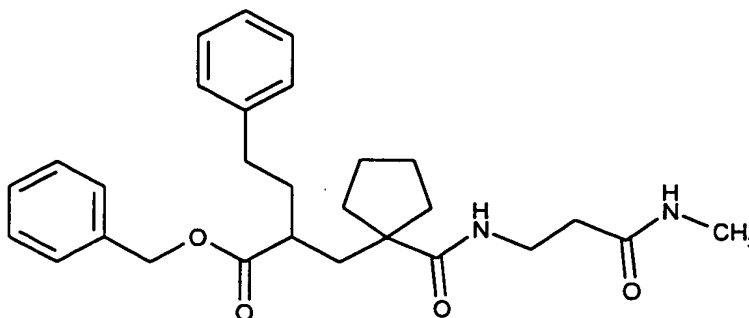
Benzyl 2-([1-([3-(2-Oxo-1-pyrrolidinyl)propyl]amino)carbonylcyclopentyl]-methyl)-4-phenylbutanoate



- 5 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.06g, 5.53mmol), 1-hydroxybenzotriazole hydrate (0.60g, 4.44mmol) and 4-methylmorpholine (0.56g, 5.54mmol) were added sequentially to a cooled solution of 1-{2-[(benzyloxy)carbonyl]-4-phenylbutyl}cyclopentane-carboxylic acid (EP 274234) (1.5g, 3.94mmol) in dry dichloromethane (15ml) at room temperature, followed by N-(3-aminopropyl)-2-pyrrolidinone (0.56g, 3.94mmol), and the reaction stirred at room temperature for 18 hours. The mixture was washed with water, 2N hydrochloric acid, saturated aqueous sodium bicarbonate solution, and then dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The residual yellow oil was purified by column chromatography on silica gel using ethyl acetate:pentane (50:50) as the eluant to provide the title compound as a clear gum, 800mg, 40%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) d : 1.37-2.20 (m, 16H), 2.34-2.58 (m, 5H), 2.92-3.46 (m, 6H), 5.07 (d, 1H), 5.18 (d, 1H), 6.98-7.47 (m, 10H).

Preparation 68

20 Benzyl 2-([1-([3-(methylamino)-3-oxopropyl]amino)carbonyl]cyclopentyl)methyl]-4-phenylbutanoate



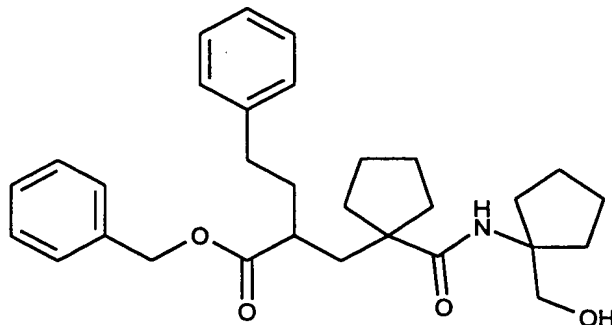
1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (122mg, 0.64mmol), 1-hydroxybenzotriazole hydrate (86mg, 0.64mmol) and 4-methylmorpholine (173μl,

1.59mmol) were added sequentially to a cooled solution of 1-{2-[(benzyloxy)carbonyl]-4-phenylbutyl}cyclopentane-carboxylic acid (EP 274234) (202mg, 0.53mmol) in N,N-dimethylformamide (5ml) at room temperature, followed by the amine hydrochloride from preparation 23 (146mg, 1.06mmol), and the reaction stirred at 90°C for 18 hours. The cooled solution was concentrated under reduced pressure and the residue partitioned between water (20ml) and ethyl acetate (100ml). The layers were separated, the organic phase washed with water (3x30ml), brine (25ml) dried (MgSO<sub>4</sub>), and evaporated under reduced pressure to give a clear oil. The crude product was purified by column chromatography on silica gel using dichloromethane:methanol (98:2) as eluant to afford the title compound as a colourless oil, 162mg, 67%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.38-1.53 (m, 2H), 1.53-1.96 (m, 8H), 2.02 (m, 2H), 2.27 (t, 2H), 2.46 (m, 3H), 2.76 (d, 3H), 3.44 (m, 2H), 5.13 (s, 2H), 5.79 (bs, 1H), 6.38 (m, 1H), 7.06 (d, 2H), 7.18 (m, 1H), 7.22 (m, 2H), 7.38 (m, 5H); LRMS : m/z 465.5 (MH<sup>+</sup>).

15

#### Preparation 69

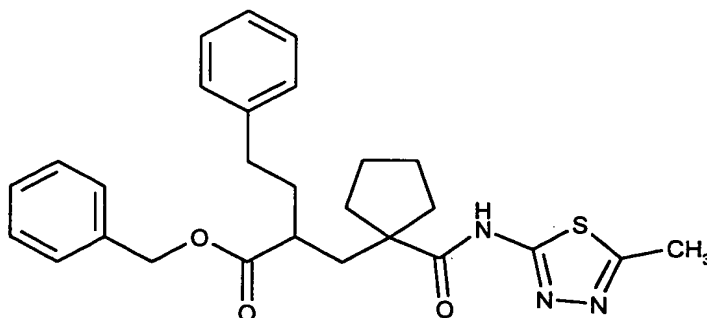
Benzyl 2-{[1-([1-(hydroxymethyl)cyclopentyl]amino)carbonyl]cyclopentyl[methyl]-4-phenylbutanoate



The title compound was obtained as a crystalline solid (48%) from 1-{2-[(benzyloxy)carbonyl]-4-phenylbutyl}cyclopentane-carboxylic acid (EP 274234) and 1-amino-1-cyclopentanemethanol, following a similar procedure to that described in preparation 68, except the reaction mixture was stirred at room temperature for 18 hours, and the crude product purified by column chromatography on silica gel using ethyl acetate:pentane as eluant; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.38 (m, 2H), 1.50-1.95 (m, 16H), 2.01 (m, 2H), 2.45 (m, 3H), 3.49 (dd, 1H), 3.60 (dd, 1H), 4.58 (m, 1H), 5.10 (s, 2H), 5.67 (s, 1H), 7.01 (d, 2H), 7.14 (m, 1H), 7.20 (m, 2H), 7.36 (m, 5H); LRMS : m/z 478.3 (MH<sup>+</sup>).

#### Preparation 70

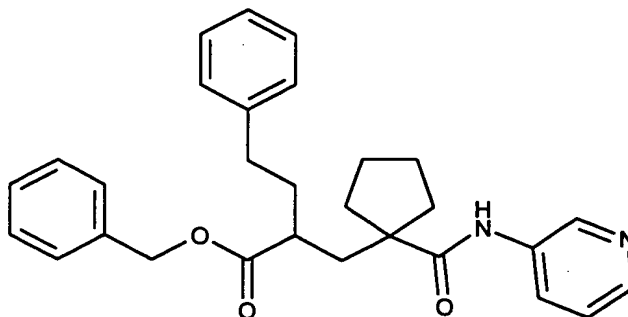
Benzyl 2-[(1-[(5-methyl-1,3,4-thiadiazol-2-yl)amino]carbonyl)cyclopentyl)methyl]-4-phenylbutanoate



The title compound was obtained as a clear oil in 74% yield from 1-{2-  
 5 [(benzyloxy)carbonyl]-4-phenylbutyl}cyclopentane-carboxylic acid (EP 274234) and  
 2-amino-5-methyl-1,3,4-thiadiazole, following a similar procedure to that described in  
 preparation 68; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.58-1.76 (m, 7H), 1.83-1.98 (m, 3H),  
 2.03 (m, 1H), 2.20 (m, 1H), 2.35 (m, 1H), 2.44 (m, 3H), 2.65 (s, 3H), 5.02 (dd, 2H),  
 7.00 (d, 2H), 7.15 (m, 1H), 7.19 (m, 2H), 7.35 (m, 5H); LRMS : m/z 478.7 (MH<sup>+</sup>).

Preparation 71

Benzyl 4-phenyl-2-[(1-[(3-pyridinylamino)carbonyl]cyclopentyl)methyl]butanoate



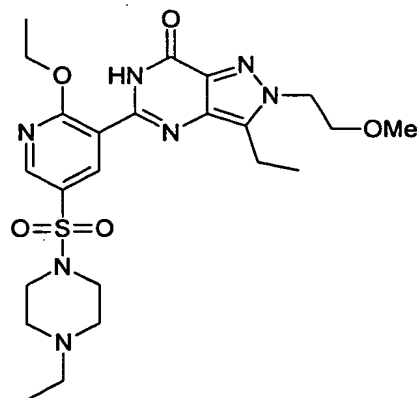
Oxalyl chloride (2.29ml, 26.3mmol) was added to a solution of 1-{2-  
 15 [(benzyloxy)carbonyl]-4-phenylbutyl}cyclopentane-carboxylic acid (EP 274234) (5.0g,  
 13.14mmol) and N,N-dimethylformamide (2 drops) in dichloromethane (25ml), and  
 the solution stirred for 2.5 hours. The mixture was evaporated under reduced  
 pressure, the residue azeotroped with dichloromethane to give a yellow oil. This was  
 then dissolved in dichloromethane (50ml) and a solution of this acid chloride (10ml,  
 20 2.45mmol) was added to an ice-cooled solution of triethylamine (248mg, 2.45mmol)  
 and 3-aminopyridine (253mg, 2.70mmol) in dry dichloromethane (10ml), and the  
 reaction stirred at room temperature for 18 hours. The solution was washed with  
 water (3x), dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The crude  
 product was purified by column chromatography on silica gel using ethyl



acetate:hexane (40:60) as eluant, and repeated using an elution gradient of ether:hexane (90:10 to 100:0). The product was crystallised from ethyl acetate:hexane to afford the title compound, 740mg, 66%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300MHz)  $\delta$ : 1.38-2.07 (m, 10H), 2.10-2.37 (m, 2H), 2.42-2.63 (m, 3H), 5.02 (s, 2H), 6.94-7.44 (m, 10H), 7.50 (s, 1H), 8.03 (d, 1H), 8.36 (d, 1H), 8.52 (s, 1H).

### PDE5i Example 1

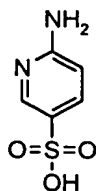
#### 10 2-(Methoxyethyl)-5-[2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one



A mixture of the product from stage j) below (0.75mmol), potassium bis(trimethylsilyl)amide (298mg, 1.50mmol) and ethyl acetate (73 microlitres, 0.75mmol) in ethanol (10ml) was heated at 120°C in a sealed vessel for 12 hours. The cooled mixture was partitioned between ethyl acetate and aqueous sodium bicarbonate solution, and the layers separated. The organic phase was dried ( $\text{MgSO}_4$ ), and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane:methanol (98:2) as eluant to afford the title compound, 164mg; Found : C, 53.18; H, 6.48; N, 18.14;  $\text{C}_{23}\text{H}_{33}\text{N}_7\text{O}_5\text{S}; 0.20\text{C}_2\text{H}_5\text{CO}_2\text{CH}_3$  requires C, 53.21; H, 6.49; N, 18.25%;  $\delta$  ( $\text{CDCl}_3$ ) : 1.04 (3H, t), 1.40 (3H, t), 1.58 (3H, t), 2.41 (2H, q), 2.57 (4H, m), 3.08 (2H, q), 3.14 (4H, m), 3.30 (3H, s), 3.92 (2H, t), 4.46 (2H, t), 4.75 (2H, q), 8.62 (1H, d), 9.04 (1H, d), 10.61 (1H, s); LRMS :  $m/z$  520 ( $\text{M}+1$ ) $^+$ ; mp 161-162°C.

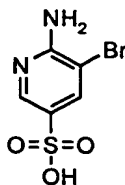
### Preparation of Starting Materials for PDE5i Example 1

#### a) Pyridine-2-amino-5-sulphonic acid



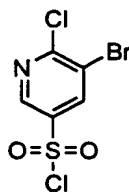
2-Aminopyridine (80g, 0.85mol) was added portionwise over 30 minutes to oleum (320g) and the resulting solution heated at 140°C for 4 hours. On cooling, the reaction was poured onto ice (200g) and the mixture stirred in an ice/salt bath for a further 2 hours. The resulting suspension was filtered, the solid washed with ice water (200ml) and cold IMS (200ml) and dried under suction to afford the title compound as a solid, 111.3g; LRMS : m/z 175 (M+1)<sup>+</sup>.

b) Pyridine-2-amino-3-bromo-5-sulphonic acid



Bromine (99g, 0.62mol) was added dropwise over an hour, to a hot solution of the product from stage a) (108g, 0.62mol) in water (600ml) so as to maintain a steady reflux. Once the addition was complete the reaction was cooled and the resulting mixture filtered. The solid was washed with water and dried under suction to afford the title compound, 53.4g;  $\delta$  (DMSO-d<sub>6</sub>, 300MHz): 8.08 (1H, s), 8.14 (1H, s); LRMS : m/z 253 (M)<sup>+</sup>.

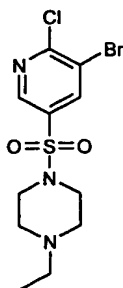
c) Pyridine-3-bromo-2-chloro-5-sulphonyl chloride



A solution of sodium nitrite (7.6g, 110.0mmol) in water (30ml) was added dropwise to an ice-cooled solution of the product from stage b) (25.3g, 100.0mmol) in aqueous hydrochloric acid (115ml, 20%), so as to maintain the temperature below 6°C. The reaction was stirred for 30 minutes at 0°C and for a further hour at room temperature. The reaction mixture was evaporated under reduced pressure and the residue dried under vacuum at 70°C for 72 hours. A mixture of this solid, phosphorus pentachloride (30.0g, 144mmol) and phosphorus oxychloride (1ml, 10.8mmol) was heated at 125°C

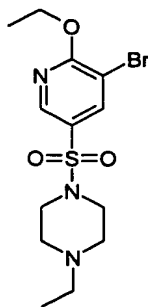
for 3 hours, and then cooled. The reaction mixture was poured onto ice (100g) and the resulting solid filtered, and washed with water. The product was dissolved in dichloromethane, dried ( $\text{MgSO}_4$ ), and evaporated under reduced pressure to afford the title compound as a yellow solid, 26.58g;  $\delta$  ( $\text{CDCl}_3$ , 300MHz) : 8.46 (1H, s), 8.92 (1H, s).

d) 3-Bromo-2-chloro-5-(4-ethylpiperazin-1-ylsulphonyl)pyridine



A solution of 1-ethylpiperazine (11.3ml, 89.0mmol) and triethylamine (12.5ml, 89.0mmol) in dichloromethane (150ml) was added dropwise to an ice-cooled solution of the product from stage c) (23.0g, 79.0mmol) in dichloromethane (150ml) and the reaction stirred at  $0^\circ\text{C}$  for an hour. The reaction mixture was concentrated under reduced pressure and the residual brown oil was purified by column chromatography on silica gel, using an elution gradient of dichloromethane:methanol (99:1 to 97:3) to afford the title compound as an orange solid, 14.5g;  $\delta$  ( $\text{CDCl}_3$ , 300MHz) : 1.05 (3H, t), 2.42 (2H, q), 2.55 (4H, m), 3.12 (4H, m), 8.24 (1H, s), 8.67 (1H, s).

e) 3-Bromo-2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridine

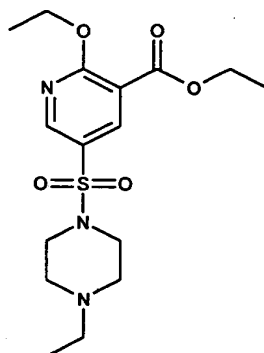


A mixture of the product from stage d) (6.60g, 17.9mmol) and sodium ethoxide (6.09g, 89.55mmol) in ethanol (100ml) was heated under reflux for 18 hours, then cooled. The reaction mixture was concentrated under reduced pressure, the residue partitioned between water (100ml) and ethyl acetate (100ml), and the layers separated. The aqueous phase was extracted with ethyl acetate (2x100ml), the combined organic solutions dried ( $\text{MgSO}_4$ ) and evaporated under reduced pressure

to afford the title compound as a brown solid, 6.41g; Found : C, 41.27; H, 5.33; N, 11.11.  $C_{13}H_{20}BrN_3O_3S$  requires C, 41.35; H, 5.28; N, 10.99%;  $\delta$  ( $CDCl_3$ , 300MHz) : 1.06 (3H, t), 1.48 (3H, t), 2.42 (2H, q), 2.56 (4H, m), 3.09 (4H, m), 4.54 (2H, q), 8.10 (1H, s), 8.46 (1H, s); LRMS : m/z 378, 380 (M+1)<sup>+</sup>.

5

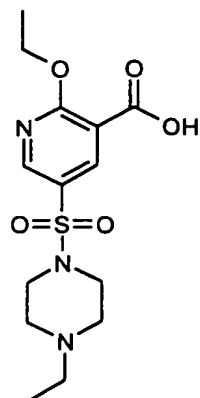
f) Pyridine 2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)-3-carboxylic acid ethyl ester



A mixture of the product from stage e) (6.40g, 16.92mmol), triethylamine (12ml, 86.1mmol), and palladium (0) tris(triphenylphosphine) in ethanol (60ml) was heated at 100°C and 200 psi, under a carbon monoxide atmosphere, for 18 hours, then cooled. The reaction mixture was evaporated under reduced pressure and the residue purified by column chromatography on silica gel, using an elution gradient of dichloromethane:methanol (100:0 to 97:3) to afford the title compound as an orange oil, 6.2g;  $\delta$  ( $CDCl_3$ , 300MHz) : 1.02 (3H, t), 1.39 (3H, t), 1.45 (3H, t), 2.40 (2H, q), 2.54 (4H, m), 3.08 (4H, m), 4.38 (2H, q), 4.55 (2H, q), 8.37 (1H, s), 8.62 (1H, s); LRMS : m/z 372 (M+1)<sup>+</sup>.

15

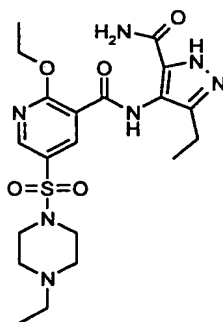
g) Pyridine 2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)-3-carboxylic acid



20

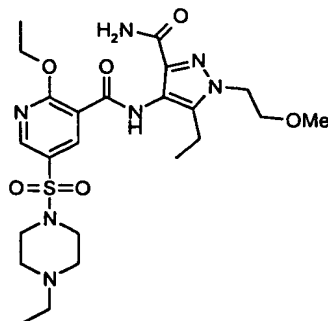
A mixture of the product from stage f) (4.96g, 13.35mmol) and aqueous sodium hydroxide solution (25ml, 2N, 50.0mmol) in ethanol (25ml) was stirred at room temperature for 2 hours. The reaction mixture was concentrated under reduced pressure to half its volume, washed with ether and acidified to pH 5 using 4N hydrochloric acid. The aqueous solution was extracted with dichloromethane (3x30ml), the combined organic extracts dried (MgSO<sub>4</sub>) and evaporated under reduced pressure to afford the title compound as a tan coloured solid, 4.02g;  $\delta$  (DMSO-d<sub>6</sub>, 300MHz) : 1.18 (3H, t), 1.37 (3H, t), 3.08 (2H, q), 3.17-3.35 (8H, m), 4.52 (2H, q), 8.30 (1H, s), 8.70 (1H, s).

h) 4-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-ylcarboxamido]-1H-3-ethylpyrazole-5-carboxamide



A solution of 4-amino-3-ethyl-1H-pyrazole-5-carboxamide (WO 9849166, preparation 8) (9.2g, 59.8mmol) in N,N-dimethylformamide (60ml) was added to a solution of the product from stage g) (21.7g, 62.9mmol), 1-hydroxybenzotriazole hydrate (10.1g, 66.0mmol) and triethylamine (13.15ml, 94.3mmol) in dichloromethane (240ml). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (13.26g, 69.2mmol) was added and the reaction stirred at room temperature for 6 hours. The dichloromethane was removed under reduced pressure, the remaining solution poured into ethyl acetate (400ml), and this mixture washed with aqueous sodium bicarbonate solution (400ml). The resulting crystalline precipitate was filtered, washed with ethyl acetate and dried under vacuum, to afford the title compound, as a white powder, 22g;  $\delta$  (CDCl<sub>3</sub>+1 drop DMSO-d<sub>6</sub>) 0.96 (3H, t), 1.18 (3H, t), 1.50 (3H, t), 2.25-2.56 (6H, m), 2.84 (2H, q), 3.00 (4H, m), 4.70 (2H, q), 5.60 (1H, br s), 6.78 (1H, br s), 8.56 (1H, d), 8.76 (1H, d), 10.59 (1H, s), 12.10-12.30 (1H, s); LRMS: m/z 480 (M+1)<sup>+</sup>.

i) 2-Methoxyethyl-4-[2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-ylcarboxamido]-3-ethylpyrazole-5-carboxamide

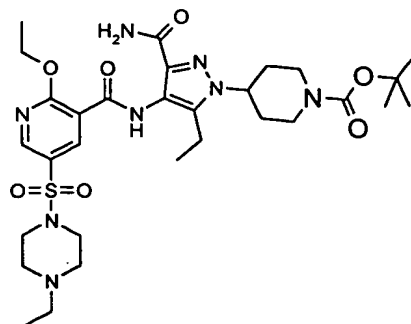


- 2-ethoxy-5-(4-ethyl-1-piperazinylsulfonyl)nicotinic acid (2.31 kg, 6.73 Mol) was suspended in ethyl acetate (16.2 L) and 1,1-carbonyldimidazole (1.09 kg, 6.73 Mol) was added at room temperature. The reaction mixture was heated at 45°C for 40 minutes and then the reaction was stirred for a further 40 minutes at reflux. After cooling to ambient temperature 4-amino-5-ethyl-1-(2-methoxyethyl)-1H-pyrazole-3-carboxamide (1.5 kg, 7.06 Mol) was added to the cooled mixture, and the reaction stirred for a further 15 hours under reflux. The mixture was cooled filtered and the filter cake was washed with 90% water / 10% ethyl acetate, (2 mL /g) to afford *N*-[3-carbamoyl-5-ethyl-1-(2-methoxyethyl)-1H-pyrazol-4-yl]-2-ethoxy-5-(4-ethyl-1-piperazinyl sulfonyl) nicotinamide as an off white crystalline solid, 3.16 kg, 88%. m.p. = 156°C. Found: C, 51.33; H, 6.56; N, 18.36. C<sub>23</sub>H<sub>35</sub>N<sub>7</sub>O<sub>6</sub>S requires C, 51.40; H, 6.53; N, 18.25%.
- $\delta$ (CDCl<sub>3</sub>): 1.04 (3H, t), 1.22 (3H, t), 1.60 (3H, t), 2.44 (2H, q), 2.54 (4H, m), 2.96 (2H, q), 3.12 (4H, m), 3.36 (3H, s), 3.81 (2H, t), 4.27 (2H, t), 4.80(2H, q), 5.35(1H, s), 6.68 (1H, s), 8.66 (1H, d), 8.86 (1H, d), 10.51 (1H, s).

LRMS: m/z = 539 (M+1)<sup>+</sup>

#### Preparation of Starting Materials for PDE5i Example 2

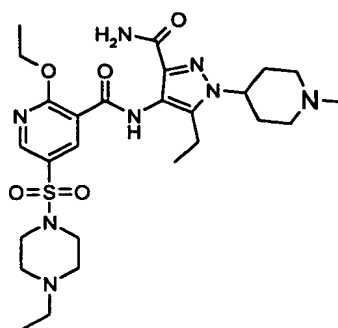
- a) 2-(1-*tert*-Butoxycarbonylpiperidin-4-yl)-4-[2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-ylcarboxamido]-3-ethylpyrazole-5-carboxamide



Sodium hydride (64mg, 60% dispersion in mineral oil, 1.6mmol) was added to a solution of the product from Example 1, stage h) (1.46mmol) in tetrahydrofuran (10ml), and the solution stirred for 10 minutes. *tert*-Butyl 4-[(methylsulphonyl)oxy]-1-piperidinecarboxylate (WO 9319059) (1.60mmol) was added and the reaction stirred at 60°C for 3 days. The cooled mixture was partitioned between ethyl acetate and aqueous sodium bicarbonate solution, and the phases separated. The aqueous layer was extracted with ethyl acetate, the combined organic solutions dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using dichloromethane:methanol (98:2) as eluant to afford the title compound as a white foam, 310 mg;  $\delta$  (CDCl<sub>3</sub>) 1.02 (3H, t), 1.23 (3H, t), 1.49 (9H, s), 1.57 (3H, m), 1.93 (2H, m), 2.16 (2H, m), 2.40 (2H, q), 2.54 (4H, m), 2.82-2.97 (4H, m), 3.10 (4H, m), 4.30 (3H, m), 4.79 (2H, q), 5.23 (1H, s), 6.65 (1H, s), 8.63 (1H, d), 8.82 (1H, d), 10.57 (1H, s).

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b) 4-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-ylcarboxamido]-3-ethyl-2-(1-methylpiperidin-4-yl)pyrazole-5-carboxamide



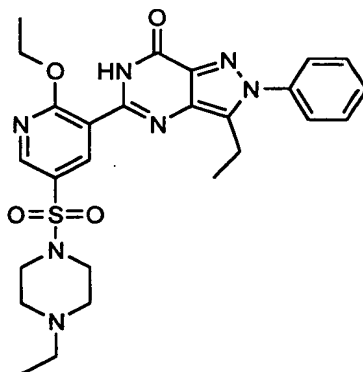
Trifluoroacetic acid (1.5ml) was added to a solution of the product from stage a) above (320mg, 0.48mmol) in dichloromethane (2ml) and the solution stirred at room temperature for 2 ½ hours. The reaction mixture was evaporated under reduced pressure and the residue triturated well with ether and dried under vacuum, to provide a white solid. Formaldehyde (217 microlitres, 37% aqueous, 2.90mmol) was added to a solution of the intermediate amine in dichloromethane (8ml), and the

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solution stirred vigorously for 30 minutes. Acetic acid (88 microlitres, 1.69mmol) was added, the solution stirred for a further 30 minutes, then sodium triacetoxyborohydride (169mg, 0.80mmol) was added and the reaction stirred at room temperature for 16 hours. The reaction mixture was poured into aqueous sodium bicarbonate solution, and extracted with ethyl acetate. The combined organic extracts were dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using dichloromethane:methanol:0.88 ammonia (91.75:7.5:0.75) as eluant to afford the title compound, 70mg;  $\delta$  (CDCl<sub>3</sub>) 1.02 (3H, t), 1.22 (3H, t), 1.58 (3H, t), 1.92 (2H, m), 2.14 (2H, m), 2.25-2.45 (7H, m), 2.54 (4H, m), 2.91 (2H, q), 2.99-3.16 (6H, m), 4.08 (1H, m), 4.78 (2H, q), 5.11 (1H, br s), 6.65 (1H, br s), 8.63 (1H, d), 8.83 (1H, d), 10.53 (1H, s).

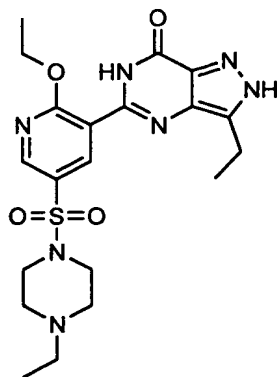
### PDE5i Example 3

5-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-phenyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one



Pyridine (0.1ml, 1.08mmol) was added to a mixture of the product from stage a) below (250mg, 0.54mmol), copper (II) acetate monohydrate (145mg, 0.72mmol), benzeneboronic acid (132mg, 1.08mmol) and 4Å molecular sieves (392mg) in dichloromethane (5ml), and the reaction stirred at room temperature for 4 days. The reaction mixture was filtered and the filtrate evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using dichloromethane:methanol:0.88 ammonia (97:3:0.5) as eluant, and triturated with ether:hexane. The resulting solid was filtered and recrystallised from isopropanol:dichloromethane to give the title compound as a solid, 200mg,  $\delta$  (CDCl<sub>3</sub>) 1.02 (3H, t), 1.47 (3H, t), 1.60 (3H, t), 2.42 (2H, q), 2.58 (4H, m), 3.10 (2H, q), 3.17 (4H, m), 4.76 (2H, q), 7.40 (1H, m), 7.51 (2H, m), 7.80 (2H, d), 8.67 (1H, d), 9.16 (1H, s), 10.90 (1H, s); LRMS : m/z 538 (M+1)<sup>+</sup>.

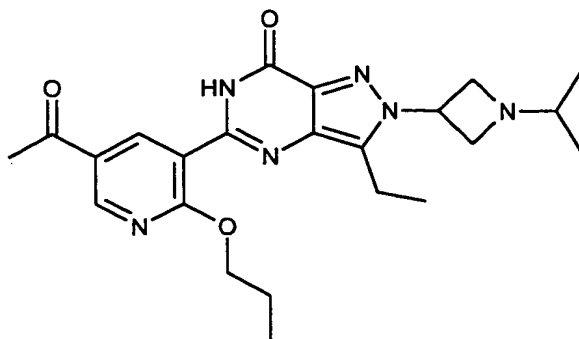


Preparation of Starting Materials for PDE5i Example 3a) 5-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

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Potassium bis(trimethylsilyl)amide (8.28g, 41.6mmol) was added to a solution of the product from Example 1, stage h) (10.0g, 20.8mmol) and ethyl acetate (2ml, 20mmol) in ethanol (160ml), and the reaction mixture heated at 120°C for 12 hours in a sealed vessel. The cooled mixture was evaporated under reduced pressure and the residue was purified by column chromatography on silica gel using dichloromethane:methanol:0.88 ammonia (95:5:0.5) as eluant, to give the title compound, 3.75g;  $\delta$  (CDCl<sub>3</sub>) 1.03 (3H, t), 1.42 (3H, t), 1.60 (3H, t), 2.42 (2H, q), 2.58 (4H, m), 3.02 (2H, q), 3.16 (4H, m), 4.78 (2H, q), 8.66 (1H, d), 9.08 (1H, d), 11.00 (1H, s) 11.05-11.20 (1H, br s), LRMS : m/z 462 (M+1)<sup>+</sup>.

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PDE5i Example 45-(5-Acetyl-2-propoxy-3-pyridinyl)-3-ethyl-2-(1-isopropyl-3-azetidiny)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

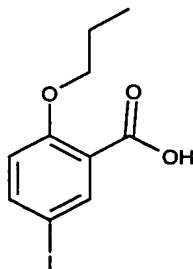
The product from stage h) below (0.23 mmol) was dissolved in dichloromethane (10 ml) and acetone (0.01 ml) was added. After 30 min stirring sodium triacetoxymethylborohydride (0.51 mmol) was added and stirring continued for 14 h.

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Further acetone (0.01 ml) and sodium triacetoxymethylborohydride (0.51 mmol) were added and stirring continued for a further 4.5 h. Starting material still remained so further acetone (0.01 ml) and sodium triacetoxymethylborohydride (0.51 mmol) were added and stirring continued for a further 18 h. The reaction mixture was diluted with  
 5 dichloromethane, washed with sodium bicarbonate solution then brine, dried ( $\text{MgSO}_4$ ) and concentrated. Purification by flash column chromatography (elution with 94:6:0.6 dichloromethane/methanol/0.88 ammonia) gave the product as a solid, M.p. 162.8-163.6°C;  $^1\text{H}$  NMR (400MHz, MeOD):  $\delta$  = 1.00 (app. d, 9H), 1.30 (t, 3H), 1.84 (app. q, 2H), 2.60 (s, 3H), 2.62-2.72 (m, 1H), 3.00-3.10 (q, 2H), 3.75 (t, 2H),  
 10 3.90 (t, 2H), 4.50 (t, 2H), 5.25 (t, 1H), 8.70 (s, 1H), 8.90 (s, 1H); LRMS (TSP – positive ion) 439 ( $\text{MH}^+$ ); Anal. Found C, 61.92; H, 6.84; N, 18.70 Calcd for  $\text{C}_{23}\text{H}_{30}\text{O}_3\text{N}_6 \cdot 0.1\text{CH}_2\text{Cl}_2$ : C, 62.07; H, 6.81; N, 18.80.

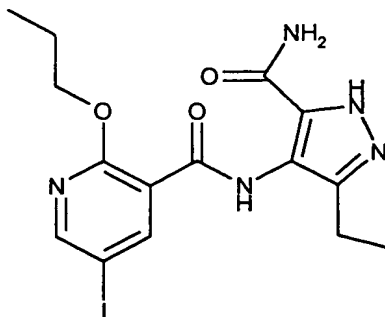
#### Preparation of Starting Materials for PDE5i Example 4

##### 15 a) 2-Propoxy-5-iodonicotinic acid



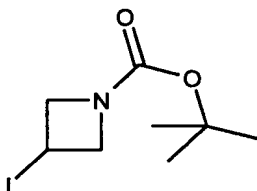
*N*-Iodosuccinamide (18.22 g, 0.08 mol), trifluoroacetic acid (100 ml) and trifluoroacetic anhydride (25 ml) were added to 2-propoxynicotinic acid (0.054 mol). The mixture was refluxed for 2.5 h, cooled and the solvents evaporated. The residue  
 20 was extracted from water with ethyl acetate and the organics washed with water (twice) and brine (twice), dried ( $\text{MgSO}_4$ ) and concentrated. The red residue was redissolved in ethyl acetate washed with sodium thiosulfate solution (twice), water (twice), brine (twice), redried ( $\text{MgSO}_4$ ) and concentrated to give the desired product as a solid;  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta$  = 1.05 (t, 3H), 1.85-2.0 (m, 2H), 4.5 (t, 2H),  
 25 8.5 (s, 1H), 8.6 (s, 1H); Analysis: found C, 35.16; H, 3.19; N, 4.46. Calcd for  $\text{C}_9\text{H}_{10}\text{INO}_3$ : C, 35.19; H, 3.28; N, 4.56%.

##### b) *N*-[3-(Aminocarbonyl)-5-ethyl-1*H*-pyrazol-4-yl]-5-iodo-2-propoxy-nicotinamide



Oxalyl chloride (15.9 mmol) was added to a stirred solution of the product from stage a) (3.98 mmol) in dichloromethane (20 ml) and 3 drops *N,N*-dimethylformamide added. After 2.5 h the solvent was evaporated and the residue azeotrope 3 times with dichloromethane. The residue was resuspended in dichloromethane (4 ml) and added to a stirred mixture 4-amino-3-ethyl-1*H*-pyrazole-5-carboxamide (prepared as described in WO 98/49166) (3.58 mmol) and triethylamine (7.97 mmol) in dichloromethane (10 ml). After 1 h the solvent was evaporated and the residue partitioned between ethyl acetate and water. The organic phase was separated and washed with 2N HCl (twice), sodium bicarbonate solution (twice) and brine before being dried (MgSO<sub>4</sub>) and concentrated. The product was triturated with ether and filtered to give the title product as a solid. The mother liquor was concentrated and purified by flash column chromatography (elution with 80% ethyl acetate : hexane) to give further product; <sup>1</sup>H NMR (300 MHz, d<sub>4</sub>-MeOH): δ = 1.0 (t, 3H), 1.25 (t, 3H), 1.85-2.0 (m, 2H), 2.8 (q, 2H), 4.5 (t, 2H), 8.5 (s, 1H), 8.6 (s, 1H); LRMS (TSP) 444 (MH<sup>+</sup>).

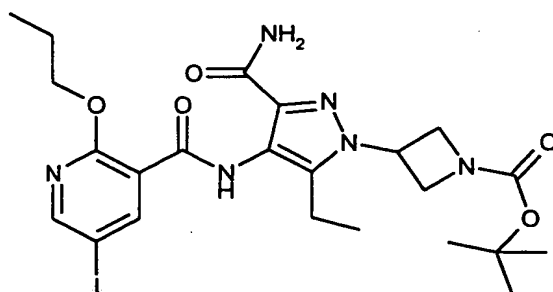
c) *tert*-Butyl 3-iodo-1-azetidinecarboxylate



A mixture of *tert*-butyl 3-[(methylsulfonyl)oxy]-1-azetidinecarboxylate (prepared as described in *Synlett* 1998, 379; 5.0 g, 19.9 mmol), and potassium iodide (16.5 g, 99.4 mmol) in *N,N*-dimethylformamide (25 ml), was heated at 100°C for 42 h. The cooled mixture was partitioned between water and ethyl acetate, and the layers separated. The organic phase was dried over MgSO<sub>4</sub>, concentrated under reduced pressure and the residue azeotrope with xylene. The crude product was purified by flash column chromatography (dichloromethane as eluant) to give the title compound, 3.26 g; <sup>1</sup>H

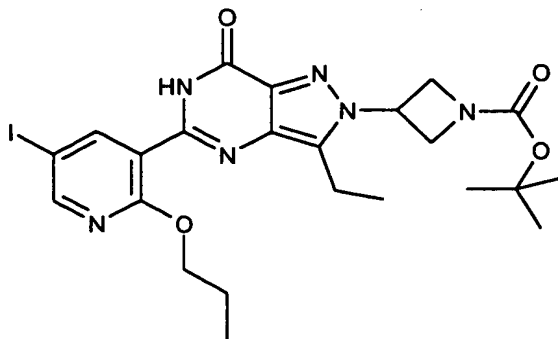
NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  = 1.43 (s, 9H), 4.28 (m, 2H), 4.46 (m, 1H), 4.62 (m, 2H); LRMS (TSP) 284 (MH)<sup>+</sup>

d) tert-Butyl 3-(3-(aminocarbonyl)-5-ethyl-4-((5-iodo-2-propoxy-3-pyridinyl)carbonyl)amino)-1H-pyrazol-1-yl)-1-azetidinecarboxylate



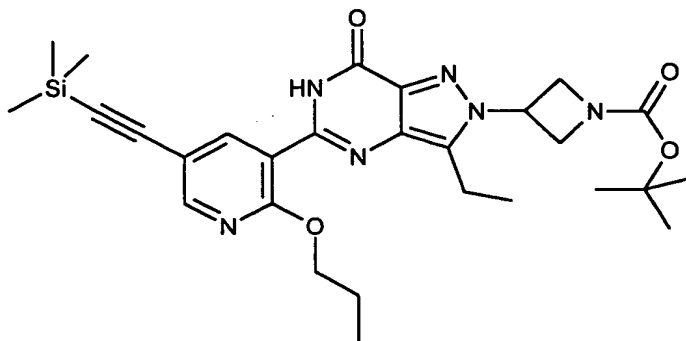
Cesium carbonate (3.59 mmol) was added to a stirred solution of the product from stage b) (1.79 mmol) and the product from stage c) (2.15 mmol) in *N,N*-dimethylformamide (10 ml) under a nitrogen atmosphere. The mixture was heated at 80°C for 24 h. The mixture was cooled and extracted from water with ethyl acetate. The organics were dried (MgSO<sub>4</sub>) and concentrated to give a brown oil. Purification by flash column chromatography (gradient elution from 100% dichloromethane to 90% dichloromethane/MeOH) gave the title product; <sup>1</sup>H NMR (400MHz, DMSO):  $\delta$  = 0.95 (t, 3H), 1.05 (t, 3H), 1.40 (s, 9H), 1.78-1.88 (m, 2H), 2.68 (q, 2H), 4.22-4.35 (m, 4H), 4.40 (t, 2H), 5.33 (t, 1H), 7.35 (bs, 1H), 7.52 (bs, 1H), 8.40 (s, 1H), 8.55 (s, 1H), 10.10 (s, 1H); LRMS (TSP – positive ion) 373.2 (MH<sup>+</sup> - BOC and I); Anal. Found C, 45.11; H, 5.07; N, 13.56 Calcd for C<sub>23</sub>H<sub>31</sub>O<sub>5</sub>N<sub>6</sub>I. 0.2 DCM: C, 45.28; H, 5.14; N, 13.66.

e) tert-Butyl 3-[3-ethyl-5-(5-iodo-2-propoxy-3-pyridinyl)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-*d*]pyrimidin-2-yl]-1-azetidinecarboxylate



The product from stage d) (28.4 mmol) was dissolved in n-propanol (200 ml), ethyl acetate (6 ml) and potassium t-butoxide (28.4 mmol) were added and the resultant mixture heated to reflux for 6h. Additional potassium t-butoxide (14.2 mmol) was added and the mixture heated for a further 2h, after which the solvent was removed *in vacuo*. The residue was partitioned between water (50 ml) and methylene chloride (100 ml) and the organic phase separated. The aqueous phase was extracted with dichloromethane (2 x 100 ml) and the combined organics dried over MgSO<sub>4</sub> and reduced to a solid. Purification by column chromatography (elution with ethyl acetate) gave the title compound; <sup>1</sup>H NMR (400MHz, CDCl<sub>3</sub>): δ = 1.05 (t, 3H), 1.30 (t, 3H), 1.43 (s, 9H), 1.87-1.96 (m, 2H), 3.00 (q, 2H), 4.34 (t, 2H), 4.49 (t, 2H), 4.60 (br s, 2H), 5.20 (t, 1H), 8.41 (d, 1H), 8.94 (s, 1H), 10.75 (br s, 1H); LRMS (TSP – positive ion) 598.1 (MNH<sub>4</sub><sup>+</sup>); Anal. Found C, 47.54; H, 5.02; N, 14.09 Calcd for C<sub>23</sub>H<sub>29</sub>O<sub>4</sub>N<sub>6</sub>: C, 47.60; H, 5.04; N, 14.48.

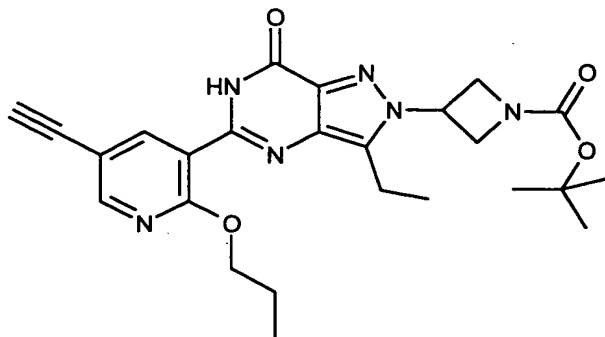
f) *tert*-Butyl 3-(3-ethyl-7-oxo-5-{2-propoxy-5-[(trimethylsilyl)ethynyl]-3-pyridinyl}-6,7-dihydro-2H-pyrazolo[4,3-*d*]pyrimidin-2-yl)-1-azetidinecarboxylate



The product from stage e) (0.25 mmol) was suspended in triethylamine (2 ml) and trimethylsilylacetylene (0.39 mmol) and acetonitrile (2 ml to try and solubilise reactants). Pd(PPh<sub>3</sub>)<sub>2</sub>Cl<sub>2</sub> (0.006 mmol) and cuprous iodide (0.006 mmol) were added and the reaction mixture stirred. After 1 h a further portion of trimethylsilylacetylene (0.19 mmol) was added and stirring continued for 2 h. The solvent was evaporated and the residue partitioned between ethyl acetate and water. The organics were washed with brine, dried (MgSO<sub>4</sub>) and concentrated. Purification by flash column chromatography (gradient elution from 100% dichloromethane to 99% dichloromethane/methanol) gave the title compound; <sup>1</sup>H NMR (400MHz, MeOD): δ = 0.25 (s, 9H), 1.05 (t, 3H), 1.31 (t, 3H), 1.44 (s, 9H), 1.87-1.96 (m, 2H), 3.00 (q, 2H), 4.33 (t, 2H), 4.52 (t, 2H), 4.54-4.80 (m, 2H), 5.18-5.25 (m, 1H), 8.32 (d, 1H), 8.74 (d,

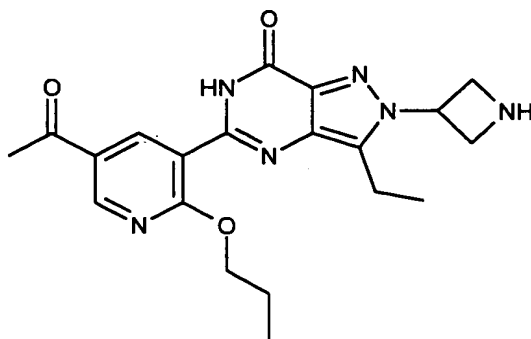
1H); LRMS (TSP – positive ion) 569 ( $\text{MNH}_4^+$ ), 452.0 ( $\text{MH}^+$ ); Anal. Found C, 60.82; H, 6.90; N, 15.15 Calcd for  $\text{C}_{28}\text{H}_{38}\text{O}_4\text{N}_6\text{Si}$ : C, 61.07; H, 6.95; N, 15.26.

g) tert-Butyl 3-[3-ethyl-5-(5-ethynyl-2-propoxy-3-pyridinyl)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl]-1-azetidinecarboxylate



Potassium fluoride (0.38 mmol) was added to a stirred solution of the product of stage f) (0.19 mmol) in aqueous *N,N*-dimethylformamide (2 ml *N,N*-dimethylformamide /0.2 ml water) at 0°C. After 10 min the reaction was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was diluted with ethyl acetate and washed with water, 1 *N* hydrochloric acid (3 times) and brine. The organic layer was dried ( $\text{MgSO}_4$ ) and concentrated to give the title compound as a solid;  $^1\text{H}$  NMR (400MHz,  $\text{CDCl}_3$ ):  $\delta$  = 1.05 (t, 3H), 1.30 (t, 3H), 1.43 (s, 9H), 1.88-2.00 (m, 2H), 3.00 (q, 2H), 3.19 (s, 1H), 4.35 (app t, 2H), 4.52 (app t, 2H), 4.60-4.80 (br s, 2H), 5.22 (t, 1H), 8.39 (s, 1H), 8.80 (s, 1H), 10.75 (br s, 1H); LRMS (TSP – positive ion) 496 ( $\text{MNH}_4^+$ ).

h) 5-(5-Acetyl-2-propoxy-3-pyridinyl)-2-(3-azetidynyl)-3-ethyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one



The product from stage g) (1.44 g, 3.0 mmol) in acetone (50 ml) and sulphuric acid (1*N*, 3 ml) was treated with mercuric sulphate (268 mg, 9.0 mmol) and heated to

reflux for 6h. The reaction mixture was concentrated to ~20 ml *in vacuo*, poured into sodium bicarbonate (sat. aq., 20ml) and extracted into methylene chloride (6 x 20 ml). Combined organics were washed with brine (20 ml), dried over MgSO<sub>4</sub>, and concentrated to a brown oil which was taken up in 40% trifluoroacetic acid in methylene chloride (50ml) and water (1 ml) and stirred for 1h at room temperature. After evaporation *in vacuo*, the residue was purified by column chromatography (eluting with 95:5:1 methylene chloride:methanol:0.88 ammonia) to afford the title compound as a white hygroscopic foam ( 1.65 g); m.p. 128.5-130.0°C; <sup>1</sup>H NMR (400MHz, MeOD):  $\delta$  = 1.00 (t, 3H), 1.30 (t, 3H), 1.79-1.90 (m, 2H), 2.60 (s, 3H), 3.00-3.10 (q, 2H), 4.50 (t, 2H), 4.60-4.70 (m, 4H), 5.65-5.78 (m, 1H), 8.65 (s, 1H), 8.90 (s, 1H); LRMS (TSP – positive ion) 397 (MH<sup>+</sup>).

#### PDE5i Example 5

5-(5-acetyl-2-butoxy-3-pyridinyl)-3-ethyl-2-(1-ethyl-3-azetidiny)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one.

The title compound from preparation 5(a) (120 mg, 0.28 mmol) and cesium carbonate (274 mg, 0.84 mmol) were dissolved in *n*-butanol (4 ml), and heated at 90°C under nitrogen with molecular sieves for 96h. The mixture was then partitioned between water (10 ml) and dichloromethane (10 ml). The organic layer was separated, and the aqueous layer extracted further with dichloromethane (3 x 15 ml). The combined organic layers were dried (MgSO<sub>4</sub>), and concentrated *in vacuo*. The crude product was purified by flash column chromatography (95:5:0.5-90:10:1 ethyl acetate:methanol:0.88 NH<sub>3</sub> as eluents), to yield the title compound as a colourless glass (77 mg, 0.18 mmol).

**m.p.** 91.6-93.7°C

**<sup>1</sup>H NMR** (400MHz, CDCl<sub>3</sub>):  $\delta$  = 1.00-1.05 (m, 6H), 1.38 (t, 3H), 1.50-1.62 (m, 2H), 1.90-2.00 (m, 2H), 2.63 (s, 3H), 2.63-2.70 (m, 2H), 3.02 (q, 2H), 3.75 (t, 2H), 3.90 (t, 2H), 4.68 (t, 2H), 5.10-5.20 (m, 1H), 8.84 (s, 1H), 9.23 (s, 1H), 10.63 (br s, 1H).

**LRMS** (TSP – positive ion) 439 (MH<sup>+</sup>)

**Anal.** Found C, 60.73; H, 7.06; N, 18.03 Calcd for C<sub>23</sub>H<sub>30</sub>O<sub>3</sub>N<sub>6</sub>·0.2MeOH·0.1 DIPE: C, 60.88; H, 7.26; N, 17.90

#### Preparation of starting materials for Example 5

5(a) 5-(5-Acetyl-2-propoxy-3-pyridinyl)-3-ethyl-2-(1-ethyl-3-azetidiny)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

Sodium cyanoborohydride (92 mg, 1.47 mmol) was added to a stirring solution of title compound from example 5(b) (500 mg, 0.98 mmol), acetaldehyde (64  $\mu$ l, 1.18 mmol) and sodium acetate (161 mg, 1.96 mmol) in methanol (10 ml) under nitrogen at room temperature. After 1h the mixture was poured into NaHCO<sub>3</sub> (sat. aq., 20 ml), and extracted with dichloromethane (3 x 15 ml). The combined organic layers were dried (MgSO<sub>4</sub>) and concentrated *in vacuo*. The crude product was purified by flash column chromatography (95:5:0.5-80:20:1 ethyl acetate:methanol:0.88 NH<sub>3</sub> as eluent) to yield the title compound as a white solid (140 mg, 0.33 mmol).

**<sup>1</sup>H NMR** (400MHz, CDCl<sub>3</sub>):  $\delta$  = 0.97 (t, 3H), 1.03 (t, 3H), 1.30 (t, 3H), 2.82-2.97 (m, 2H), 2.58-2.65 (m, 5H), 2.98 (q, 2H), 3.68 (t, 2H), 3.85 (dd, 2H), 4.58 (dd, 2H), 5.05-5.17 (m, 1H), 8.79 (s, 1H), 9.18 (s, 1H), 10.62 (br s, 1H).

**LRMS** (TSP – positive ion) 426 (MH<sup>+</sup>)

**5(b) 5-(5-Acetyl-2-propoxy-3-pyridinyl)-2-(3-azetidiny)-3-ethyl-2,6-dihydro-7H-pyrazolo[4,3-*d*]pyrimidin-7-one**

The title compound of Preparation 5(c) (1.44 g, 3.0 mmol) in acetone (50 ml) and sulphuric acid (1N, 3 ml) was treated with mercuric sulphate (268 mg, 9.0 mmol) and heated to reflux for 6h. The reaction mixture was concentrated to ~20 ml *in vacuo*, poured into sodium bicarbonate (sat. aq., 20ml) and extracted into methylene chloride (6 x 20 ml). Combined organics were washed with brine (20 ml), dried over MgSO<sub>4</sub>, and concentrated to a brown oil which was taken up in 40% trifluoroacetic acid in methylene chloride (50ml) and water (1 ml) and stirred for 1h at room temperature. After evaporation *in vacuo*, the residue was purified by column chromatography (eluting with 95:5:1 methylene chloride:methanol:0.88 ammonia) to afford the title compound as a white hygroscopic foam ( 1.65 g).

**m.p.** 128.5-130.0°C

**<sup>1</sup>H NMR** (400MHz, MeOD):  $\delta$  = 1.00 (t, 3H), 1.30 (t, 3H), 1.79-1.90 (m, 2H), 2.60 (s, 3H), 3.00-3.10 (q, 2H), 4.50 (t, 2H), 4.60-4.70 (m, 4H), 5.65-5.78 (m, 1H), 8.65 (s, 1H), 8.90 (s, 1H)

**LRMS** (TSP – positive ion) 397 (MH<sup>+</sup>)

**5(c) tert-Butyl 3-[3-ethyl-5-(5-ethynyl-2-propoxy-3-pyridinyl)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-*d*]pyrimidin-2-yl]-1-azetidinecarboxylate**

Prepared from the title compound of Preparation 5(d) by the method of Preparation 5(c)(i).



**<sup>1</sup>H NMR** (400MHz, CDCl<sub>3</sub>): δ = 1.05 (t, 3H), 1.30 (t, 3H), 1.43 (s, 9H), 1.88-2.00 (m, 2H), 3.00 (q, 2H), 3.19 (s, 1H), 4.35 (app t, 2H), 4.52 (app t, 2H), 4.60-4.80 (br s, 2H), 5.22 (t, 1H), 8.39 (s, 1H), 8.80 (s, 1H), 10.75 (br s, 1H)

**LRMS** (TSP – positive ion) 496 (MNH<sub>4</sub><sup>+</sup>).

5

5(c)(i) 5-(2-Butoxy-5-ethynyl-3-pyridinyl)-3-ethyl-2-(2-methoxyethyl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

Potassium fluoride (22 mg, 0.38 mmol) was added to a stirred solution of the title compound of Preparation 5(d)(i) (90 mg, 0.19 mmol) in aqueous *N,N*-dimethylformamide (2 mL *N,N*-dimethylformamide /0.2 mL water) at 0°C. After 10 min the reaction was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was diluted with ethyl acetate and washed with water, 1 *N* hydrochloric acid (3 times) and brine. The organic layer was dried (MgSO<sub>4</sub>) and concentrated to give the title compound as a white solid (75 mg).

**<sup>1</sup>H NMR** (400 MHz, CDCl<sub>3</sub>): δ = 1.00 (t, 3H), 1.40 (t, 3H), 1.50 (m, 2H), 1.90 (m, 2H), 3.05 (q, 2H), 3.20 (s, 1H), 3.30 (s, 3H), 3.85 (t, 2H), 4.40 (t, 2H), 4.60 (t, 2H), 8.40 (s, 1H), 8.80 (s, 1H), 10.70 (s, 1H).

**LRMS** (TSP): 396.3 (MH<sup>+</sup>).

20

4(d) *tert*-Butyl 3-(3-ethyl-7-oxo-5-{2-propoxy-5-[(trimethylsilyl)ethynyl]-3-pyridinyl}-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl)-1-azetidinecarboxylate

Prepared from the title compound of Preparation 5(e) by the method of Preparation 5(d)(i).

**<sup>1</sup>H NMR** (400MHz, MeOD): δ = 0.25 (s, 9H), 1.05 (t, 3H), 1.31 (t, 3H), 1.44 (s, 9H), 1.87-1.96 (m, 2H), 3.00 (q, 2H), 4.33 (t, 2H), 4.52 (t, 2H), 4.54-4.80 (m, 2H), 5.18-5.25 (m, 1H), 8.32 (d, 1H), 8.74 (d, 1H)

**LRMS** (TSP – positive ion) 569 (MNH<sub>4</sub><sup>+</sup>), 552.0 (MH<sup>+</sup>)

**Anal.** Found C, 60.82; H, 6.90; N, 15.15 Calcd for C<sub>28</sub>H<sub>38</sub>O<sub>4</sub>N<sub>6</sub>Si: C, 61.07; H, 6.95; N, 15.26.

30

5(d)(i) 5-(2-Butoxy-5-trimethylsilylethynyl-3-pyridinyl)-3-ethyl-2-(2-methoxy-ethyl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

The title compound from Example 1 of PCT application IB00/1430 (127 mg, 0.25 mmol) was suspended in triethylamine (2 mL) and trimethylsilylacetylene (38 mg, 0.39 mmol) and acetonitrile (2 mL). Pd(PPh<sub>3</sub>)<sub>2</sub>Cl<sub>2</sub> (5 mg, 0.006 mmol) and cuprous

35

iodide (1.2 mg, 0.006 mmol) were added and the reaction mixture stirred. After 1 h a further portion of trimethylsilylacetylene (19 mg, 0.19 mmol) was added and stirring continued for 2 h. The solvent was evaporated and the residue partitioned between ethyl acetate and water. The organics were washed with brine, dried (MgSO<sub>4</sub>) and concentrated to give a brown foam. Purification by flash column chromatography (gradient elution from 100% dichloromethane to 99% dichloromethane/methanol) gave the title compound as a light brown solid (108 mg).

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ = 0.25 (s, 9H), 1.00 (t, 3H), 1.40 (t, 3H), 1.50 (m, 2H), 1.90 (m, 2H), 3.10 (q, 2H), 3.30 (s, 3H), 3.90 (t, 2H), 4.40 (t, 2H), 4.60 (t, 2H), 8.40 (s, 1H), 8.80 (s, 1H), 10.70 (s, 1H).

LRMS (TSP): 468.3 (MH<sup>+</sup>).

5(e) tert-Butyl 3-[3-ethyl-5-(5-iodo-2-propoxy-3-pyridinyl)-7-oxo-6,7-dihydro-2H-pyrazolo[4,3-d]pyrimidin-2-yl]-1-azetidinecarboxylate

The title compound was prepared from the product of Preparation 5(f) using the method of Preparation 5(e)(i).

<sup>1</sup>H NMR (400MHz, CDCl<sub>3</sub>): δ = 1.05 (t, 3H), 1.30 (t, 3H), 1.43 (s, 9H), 1.87-1.96 (m, 2H), 3.00 (q, 2H), 4.34 (t, 2H), 4.49 (t, 2H), 4.60 (br s, 2H), 5.20 (t, 1H), 8.41 (d, 1H), 8.94 (s, 1H), 10.75 (br s, 1H)

LRMS (TSP – positive ion) 598.1 (MNH<sub>4</sub><sup>+</sup>)

Anal. Found C, 47.54; H, 5.02; N, 14.09 Calcd for C<sub>23</sub>H<sub>29</sub>O<sub>4</sub>N<sub>6</sub>I: C, 47.60; H, 5.04; N, 14.48.

5(e)(i) 3-Ethyl-5-(5-iodo-2-propoxy-3-pyridinyl)-1-[2-(4-morpholinyl)ethyl]-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one

The title compound of Preparation 48 of PCT application IB00/1430 (15.78 g, 28.4 mmol) was dissolved in n-propanol (200 ml), ethyl acetate (6 ml) and potassium t-butoxide (3.2 g, 28.4 mmol) were added and the resultant mixture heated to reflux for 6h. Additional potassium t-butoxide (1.6 g, 14.2 mmol) was added and the mixture heated for a further 2h, after which the solvent was removed *in vacuo*. The residue was partitioned between water (50 ml) and methylene chloride (100 ml) and the organic phase separated. The aqueous phase was extracted with dichloromethane (2 x 100 ml) and the combined organics dried over MgSO<sub>4</sub> and reduced to a yellow solid (~17 g). Purification by column chromatography (elution with ethyl acetate) gave the title compound (13.3 g, 24.1 mmol) together with recovered starting material (2.31 g, 4.2 mmol).

**m.p.** 175-177°C.

**<sup>1</sup>H NMR** (300 MHz, CDCl<sub>3</sub>): δ = 1.1 (t, 3H), 1.4 (t, 3H), 1.9-2.05 (m, 2H), 2.45-2.55 (m, 4H), 2.85 (t, 2H), 3.0 (q, 2H), 3.6-3.65 (m, 4H), 4.5 (t, 2H), 4.7 (t, 2H), 8.4 (s, 1H), 9.0 (s, 1H), 10.95 (br s, 1H).

5 **LRMS** (TSP) 540 (MH<sup>+</sup>).

**Analysis:** found C, 46.79; H, 5.01; N, 15.44. Calcd for C<sub>21</sub>H<sub>27</sub>N<sub>6</sub>O<sub>3</sub>I : C, 46.85; H, 5.05; N, 15.61%

10 5(f) *tert*-Butyl 3-(3-(aminocarbonyl)-5-ethyl-4-[(5-iodo-2-propoxy-3-pyridinyl)carbonyl]amino)-1*H*-pyrazol-1-yl)-1-azetidinecarboxylate

The title compound was prepared by the method of Preparation 5(f)(i) using the products from Preparations 5(g) and 5(i).

15 **<sup>1</sup>H NMR** (400MHz, DMSO): δ = 0.95 (t, 3H), 1.05 (t, 3H), 1.40 (s, 9H), 1.78-1.88 (m, 2H), 2.68 (q, 2H), 4.22-4.35 (m, 4H), 4.40 (t, 2H), 5.33 (t, 1H), 7.35 (bs, 1H), 7.52 (bs, 1H), 8.40 (s, 1H), 8.55 (s, 1H), 10.10 (s, 1H)

**LRMS** (TSP – positive ion) 373.2 (MH<sup>+</sup> - BOC and I)

**Anal.** Found C, 45.11; H, 5.07; N, 13.56 Calcd for C<sub>23</sub>H<sub>31</sub>O<sub>5</sub>N<sub>6</sub>I. 0.2 DCM: C, 45.28; H, 5.14; N, 13.66.

20

5(f)(i) *N*-{3-(Aminocarbonyl)-1-[2-dimethylamino)ethyl]-5-ethyl-1*H*-pyrazol-4-yl}-2-butoxy-5-iodonicotinamide

Cesium carbonate (1.17 g, 3.59 mmol) was added to a stirred solution of the title compound from Preparation 16 of PCT application IB00/1430 (800 mg, 1.79 mmol) and *N,N*-dimethylaminoethyl chloride hydrochloride (309 mg, 2.15 mmol) in *N,N*-dimethylformamide (10 mL) under a nitrogen atmosphere. The mixture was heated at 80°C for 24 h. The mixture was cooled and extracted from water with ethyl acetate. The organics were dried (MgSO<sub>4</sub>) and concentrated to give a brown oil. Purification by flash column chromatography (gradient elution from 100% dichloromethane to 90% dichloromethane/MeOH) gave the product as a pale brown oil (522 mg).

30 **<sup>1</sup>H NMR** (400 MHz, CDCl<sub>3</sub>): δ = 0.95 (t, 3H), 1.20 (t, 3H), 1.40 (m, 2H), 1.90 (m, 2H), 2.35 (s, 6H), 2.80 (t, 2H), 2.85 (q, 2H), 4.20 (t, 2H), 4.60 (t, 2H), 5.30 (br s, 1H), 6.60 (br s, 1H), 8.40 (s, 1H), 8.75 (s, 1H), 10.35 (s, 1H).

35 **LRMS** (TSP): 529.5 (MH<sup>+</sup>).

5(g) N-[3-(Aminocarbonyl)-5-ethyl-1H-pyrazol-4-yl]-5-iodo-2-propoxy-nicotinamide

The title compound was prepared from 2-propoxy-5-iodonicotinic acid (see Preparation 5(h) and 4-amino-3-ethyl-1H-pyrazole-5-carboxamide (prepared as described in WO 98/49166) according to the method described in Preparation 5(g)(i).

5 <sup>1</sup>H NMR (300 MHz, d<sub>4</sub>-MeOH): δ = 1.0 (t, 3H), 1.25 (t, 3H), 1.85-2.0 (m, 2H), 2.8 (q, 2H), 4.5 (t, 2H), 8.5 (s, 1H), 8.6 (s, 1H).

LRMS (TSP) 444 (MH<sup>+</sup>).

10 5(g)(i) N-[3-(Aminocarbonyl)-5-ethyl-1-(2-methoxyethyl)-1H-pyrazol-4-yl]-2-butoxy-5-iodonicotinamide

Oxalyl chloride (2 g, 15.9 mmol) was added to a stirred solution of the title compound from Preparation 4 of PCT application IB00/1430 (1.28 g, 3.98 mmol) in dichloromethane (20 mL) and 3 drops *N,N*-dimethylformamide added. After 2.5 h the solvent was evaporated and the residue azeotrope 3 times with dichloromethane.

15 The residue was resuspended in dichloromethane (4 mL) and added to a stirred mixture of the title compound of Preparation 11 from PCT application IB00/1430 (0.76 g, 3.58 mmol) and triethylamine (0.8 g, 7.97 mmol) in dichloromethane (10 mL). After 1 h the solvent was evaporated and the residue partitioned between ethyl acetate and water. The organic phase was separated and washed with 2N HCl  
20 (twice), sodium bicarbonate solution (twice) and brine before being dried (MgSO<sub>4</sub>) and concentrated. The product was triturated with ether and filtered to give 820 mg of pure product as a white solid. The mother liquor was concentrated and purified by flash column chromatography (elution with 80% ethyl acetate : hexane), to give a further 605 mg of product.

25 <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>): δ = 0.95 (t, 3H), 1.20 (t, 3H), 1.45 (m, 2H), 1.90 (m, 2H), 2.85 (q, 2H), 3.35 (s, 3H), 3.80 (t, 2H), 4.25 (t, 2H), 4.60 (t, 2H), 5.20 (br s, 1H), 6.60 (br s, 1H), 8.40 (s, 1H), 8.80 (s, 1H), 10.30 (s, 1H).

LRMS (TSP): 516.2 (MH<sup>+</sup>).

30 5(h) 2-Propoxy-5-iodonicotinic acid

The title compound was prepared from 2-propoxy nicotinic acid (prepared as described in WO 99/54333, the compound 2-n-propoxypyridine-3-carboxylic acid, Preparation 46 prepared by the process of Preparation 1) using the method of Preparation 5(h)(i).

35 <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ = 1.05 (t, 3H), 1.85-2.0 (m, 2H), 4.5 (t, 2H), 8.5 (s, 1H), 8.6 (s, 1H).

**Analysis:** found C, 35.16; H, 3.19; N, 4.46. Calcd for C<sub>9</sub>H<sub>10</sub>INO<sub>3</sub>: C, 35.19; H, 3.28; N, 4.56%

5(h)(i) 2-isoButoxy-5-iodo nicotinic acid

5 *N*-Iodosuccinamide (18.22 g, 0.08 mol), trifluoroacetic acid (100 mL) and trifluoroacetic anhydride (25 mL) were added to 2-isobutoxynicotinic acid (10.55 g, 0.054 mol). The mixture was refluxed for 2.5 h, cooled and the solvents evaporated. The residue was extracted from water with ethyl acetate and the organics washed with water (twice) and brine (twice), dried (MgSO<sub>4</sub>) and concentrated. The red  
10 residue was redissolved in ethyl acetate washed with sodium thiosulfate solution (twice), water (twice), brine (twice), redried (MgSO<sub>4</sub>) and concentrated to give the desired product as a yellow solid.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ = 1.05 (d, 6H), 2.20 (m, 1H), 4.40 (d, 2H), 8.50 (s, 1H), 8.70 (s, 1H),

15 LRMS (TSP): 322.3 (MH<sup>+</sup>).

5(i) *tert*-Butyl 3-iodo-1-azetidinecarboxylate

A mixture of *tert*-butyl 3-[(methylsulfonyl)oxy]-1-azetidinecarboxylate (prepared as described in *Synlett* 1998, 379; 5.0 g, 19.9 mmol), and potassium iodide (16.5 g, 99.4  
20 mmol) in *N,N*-dimethylformamide (25 mL), was heated at 100°C for 42 h. The cooled mixture was partitioned between water and ethyl acetate, and the layers separated. The organic phase was dried over MgSO<sub>4</sub>, concentrated under reduced pressure and the residue azeotroped with xylene. The crude product was purified by flash column chromatography (dichloromethane as eluant) to give the title compound, 3.26 g.

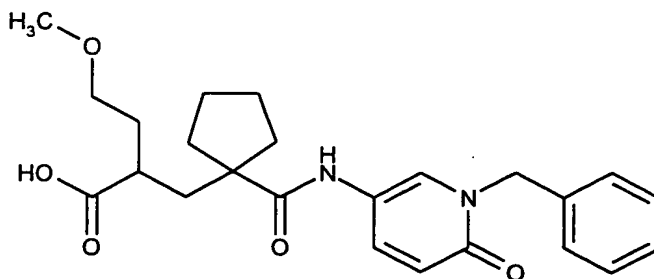
25 <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) δ = 1.43 (s, 9H), 4.28 (m, 2H), 4.46 (m, 1H), 4.62 (m, 2H).  
LRMS (TSP) 284 (MH<sup>+</sup>)

Further NEP Chemical Compound Examples

30 In the following commentary, the Preparation Examples are the synthesis of intermediates; whereas the Examples are the synthesis of the respective, compounds of the present invention.

Example 1

35 2-[(1-[(1-Benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl)cyclopentyl)methyl]-4-methoxybutanoic acid (F57)

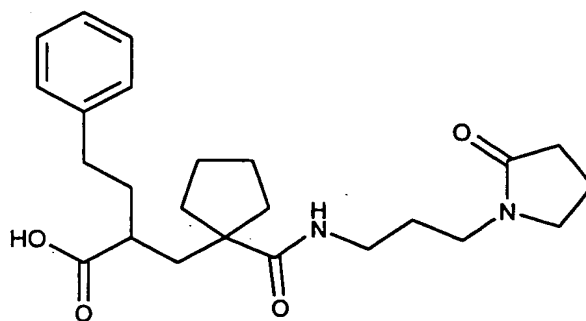


A mixture of the benzyl ester from preparation 1 (1/62) (850mg, 1.64mmol), and 5% palladium on charcoal (250mg) in 40% aqueous ethanol (21ml), was hydrogenated at 30 psi and room temperature for 30 minutes. The reaction mixture was filtered through Hyflo®, and the filtrate evaporated under reduced pressure. The residual foam was purified by column chromatography on silica gel using dichloromethane:methanol (97:3) as eluant to give the title compound as a white foam, 550mg, 79%; <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300MHz) d: 1.24-2.17 (m, 12H), 2.18-2.31 (m, 1H), 3.07 (s, 3H), 3.21 (t, 2H), 5.08 (s, 2H), 6.63 (d, 1H), 7.23-7.41 (m, 5H), 7.72 (d, 1H), 8.24 (s, 1H).

Anal. Found: C, 67.46; H, 7.18; N, 6.24. C<sub>24</sub>H<sub>30</sub>N<sub>2</sub>O<sub>5</sub> requires C, 67.58; H, 7.09; N, 6.57%.

## Example 2

2-([1-([3-(2-Oxo-1-pyrrolidiny)propyl]amino)carbonylcyclopentyl]-methyl)-4-phenylbutanoic acid. (F58)



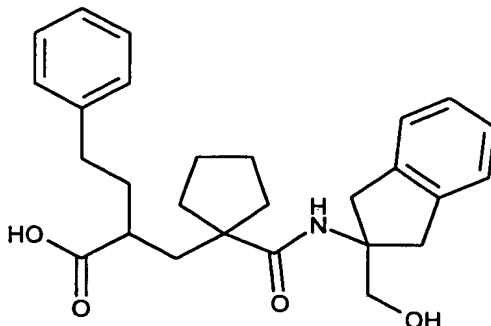
20

A mixture of the benzyl ester from preparation 3 (3/67) (780mg, 1.55mmol) and 10% palladium on charcoal (100mg) in ethanol:water (90:10 by volume), (30ml) was hydrogenated at room temperature under 60psi H<sub>2</sub> pressure for 1.5 hours. The catalyst was filtered off, and the filtrate evaporated under reduced pressure to provide the title compound as a white foam, 473mg, 74%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz)

d : 1.26-1.77 (m, 10H), 1.78-2.46 (m, 11H), 2.49-2.70 (m, 2H), 2.95-3.36 (m, 4H), 6.92-7.38 (m, 5H); Anal. Found: C, 64.05; H, 7.73; N, 6.22.  $C_{24}H_{34}N_2O_4 \cdot 0.75H_2O$  requires C, 65.88; H, 7.83; N, 6.40%.

**Example 3**

(+)-2-([1-([2-(Hydroxymethyl)-2,3-dihydro-1H-inden-2-yl]amino)carbonyl]cyclopentyl)-methyl]-4-phenylbutanoic acid (F59)



5

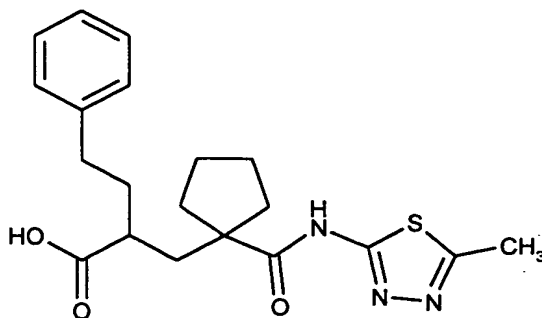
2-([1-([2-(Hydroxymethyl)-2,3-dihydro-1H-inden-2-yl]amino)carbonyl]cyclopentyl)-methyl]-4-phenylbutanoic acid (WO 9110644) may be purified by standard HPLC procedures using an AD column and hexane:isopropanol:trifluoroacetic acid (70:30:0.2) as eluant, to give the title compound of example 3, 99.5% ee;  $[\alpha]_D = +9.1^\circ$  (c = 1.76 in ethanol)

10

**Example 4**

2-([1-([5-Methyl-1,3,4-thiadiazol-2-yl]amino)carbonyl]cyclopentyl)-methyl]-4-phenylbutanoic acid (F60)

15



20

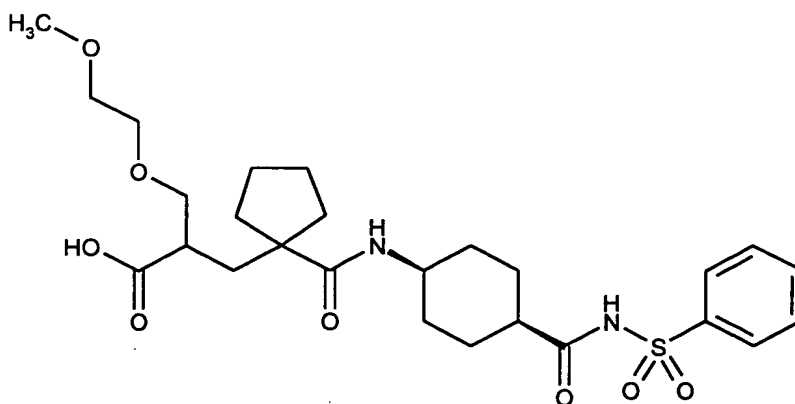
A mixture of the benzyl ester from preparation 4 (4/70) (187mg, 0.39mmol) and 10% palladium on charcoal (80mg) in ethanol (20ml) was hydrogenated at 60 psi for 18 hours. Tlc analysis showed starting material remaining, so additional 10% palladium on charcoal (100mg) was added, and the reaction continued for a further 5 hours. Tlc analysis again showed starting material remaining, so additional catalyst (100mg) was added, and hydrogenation continued for 18 hours. The mixture was filtered



through Arbocel®, and the filtrate concentrated under reduced pressure, and azeotroped with dichloromethane. The crude product was purified by chromatography on silica gel using a Biotage® column, and dichloromethane:methanol (95:5) as eluant to afford the title compound as a clear oil, 80mg, 53%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) d: 1.51-1.89 (m, 9H), 2.03 (m, 1H), 2.20 (m, 1H), 2.40 (m, 2H), 2.60 (m, 5H), 7.15-7.30 (m, 5H); LRMS : m/z 387.8 (MH<sup>+</sup>).

### Example 5

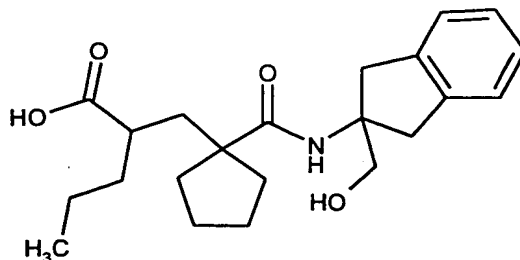
Cis-3-(2-Methoxyethoxy)-2-[(1-[(4-[(phenylsulfonyl)amino]carbonyl)cyclohexyl]-amino]carbonyl)cyclopentyl)methyl]propanoic acid (F61)



A solution of the *tert*-butyl ester from preparation 8 (8/66) (446mg, 0.75mmol) in dichloromethane (5ml) and trifluoroacetic acid (5ml) was stirred at room temperature for 18 hours. The reaction mixture was concentrated under reduced pressure, and the residue azeotroped with dichloromethane, then toluene, and finally ether, to afford the title compound as a white foam, 385mg, 95%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) d: 1.48-2.17 (m, 18H), 2.40 (s, 1H), 2.66 (s, 1H), 3.37 (s, 3H), 3.50-3.70 (m, 6H), 3.94 (s, 1H), 6.10 (d, 1H), 6.59 (s, 1H), 7.55 (t, 2H), 7.61 (m, 1H), 8.02 (d, 2H), 9.11 (s, 1H); Anal. Found: C, 54.88; H, 6.90; N, 5.04. C<sub>26</sub>H<sub>38</sub>N<sub>2</sub>O<sub>6</sub>S·1.7H<sub>2</sub>O requires C, 57.97; H, 7.11; N, 5.20%.

**Example 6**

(+)-2-[[1-([2-(Hydroxymethyl)-2,3-dihydro-1H-inden-2-yl]amino)carbonyl]cyclopentyl]-methyl]pentanoic acid (F62)



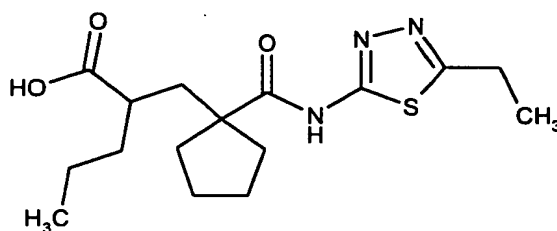
5

2-[[1-([2-(Hydroxymethyl)-2,3-dihydro-1H-inden-2-yl]amino)carbonyl]cyclopentyl]-methyl]pentanoic acid (WO 9110644) was further purified by HPLC using an AD column and hexane:isopropanol:trifluoroacetic acid (90:10:0.1) as eluant, to give the title compound of example 6, 99% ee,  $[\alpha]_D = +10.4^\circ$  (c = 0.067, ethanol).

10

**Example 7**

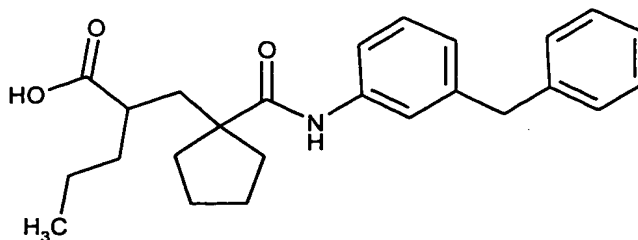
(+)-2-[[1-([5-Ethyl-1,3,4-thiadiazol-2-yl]amino)carbonyl]cyclopentyl]methyl]pentanoic acid (F63)



15

The acid from Preparation 18 (18/ex4) (824mg) was further purified by HPLC using an AD column and using hexane:iso-propanol:trifluoroacetic acid (85:15:0.2) as eluant to give the title compound of example 7 as a white foam, 386mg, 99% ee,  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz)  $\delta$ : 0.90 (t, 3H), 1.38 (m, 6H), 1.50-1.79 (m, 9H), 2.19 (m, 1H), 2.30 (m, 1H), 2.44 (m, 1H), 2.60 (m, 1H), 2.98 (q, 2H), 12.10-12.27 (bs, 1H); LRMS: m/z 338 ( $\text{MH}^+$ ); and  $[\alpha]_D = +3.8^\circ$  (c = 0.1, methanol)

20

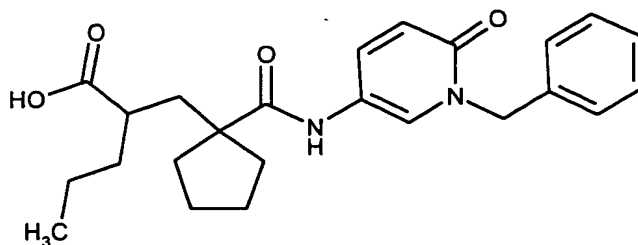
**Example 8****2-({1-[(3-Benzylanilino)carbonyl]cyclopentyl)methyl}pentanoic acid (F64)**

5

A mixture of the benzyl ester from preparation 10 (10/53) (1.3mg, 2.47mmol) and 5% palladium on charcoal (130mg) in water (10ml) and ethanol (40ml) was hydrogenated at 30 psi and room temperature for 2 hours. The reaction mixture was filtered  
 10 through Arbocel®, the filtrate concentrated under reduced pressure, and the residue triturated with dichloromethane. The residual gum was triturated with ether, then hexane, and dried at 50°C, to give the title compound as a solid, 0.79g, 81%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.95 (t, 3H), 1.24-1.51 (m, 3H), 1.58-1.80 (m, 7H), 1.88 (dd, 1H), 2.15 (m, 2H), 2.24 (m, 1H), 2.48 (m, 1H), 4.00 (s, 2H), 6.98 (d, 1H), 7.24 (m, 6H), 7.40 (m, 3H); Anal. Found: C, 75.48; H, 7.76; N, 3.59. C<sub>25</sub>H<sub>31</sub>NO<sub>3</sub>·0.25H<sub>2</sub>O  
 15 requires C, 75.44; H, 7.98; N, 3.51%.

**Example 9****2-[(1-[(1-Benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl]-cyclopentyl)methyl]-pentanoic acid (F65)**

20



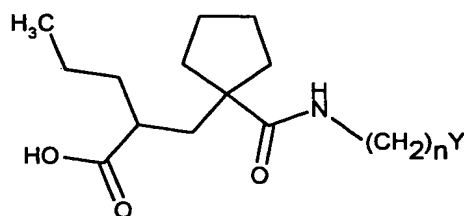
The title compound was obtained as a white foam in 51% yield from the benzyl ester  
 25 from preparation 13 (13/56), following a similar procedure to that described in Preparation 19 (19/ex21), except, the product was purified by column chromatography on silica gel, using ethyl acetate as eluant; <sup>1</sup>H NMR (CDCl<sub>3</sub>,

300MHz) d: 0.96 (t, 3H), 1.28-1.80 (m, 12H), 2.01 (m, 1H), 2.30-2.52 (m, 2H), 5.02 (dd, 2H), 6.60 (d, 1H), 7.27 (m, 5H), 7.70 (s, 1H), 8.34 (s, 1H); Anal. Found: C, 69.52; H, 7.41; N, 6.51.  $C_{24}H_{30}N_2O_4 \cdot 0.25H_2O$  requires C, 69.45; H, 7.41; N, 6.75.

5 **Example 10**

2-[(1-[(1R,3S,4R)-4-(aminocarbonyl)-3-butylcyclohexyl]amino)carbonyl]-cyclopentyl)methyl]pentanoic acid (F66)

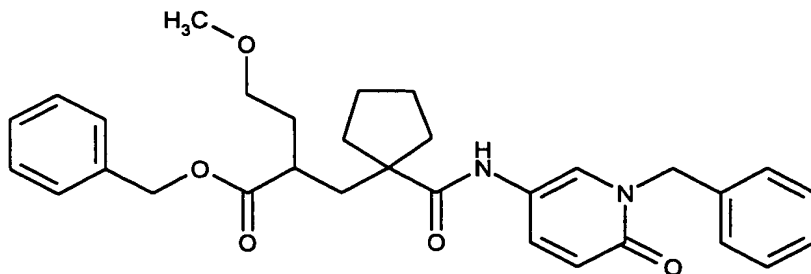
Compounds of formula ic, i.e. Compounds of general formula i where  $r^1$  is propyl, where prepared from the corresponding *tert*-butyl ester, following a similar procedure to that described in Preparation 14 (14/ex1).



(ic)

**Preparation 1 (1/62)**

15 Benzyl 2-[(1-[(1-benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl)cyclopentyl]-methyl]-4-methoxybutanoate

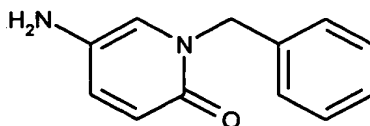


Oxalyl chloride (0.26ml, 3.0mmol) was added to an ice-cooled solution of 1-{2-[(benzyloxy)carbonyl]-4-methoxybutyl}cyclopentanecarboxylic acid (EP 274234) (1.0g, 3.0mmol) and N,N-dimethylformamide (2 drops) in dichloromethane (20ml), and the reaction stirred at room temperature for 2 hours. The solution was concentrated under reduced pressure and the residue azeotroped with dichloromethane (3x10ml). The product was dissolved in dichloromethane (20ml), then cooled in an ice-bath. The amine from preparation 2 (2/28) (600mg, 3mmol) and N-methylmorpholine (0.6ml, 5.45mmol) were added and the reaction stirred at room temperature for 18 hours. The reaction mixture was concentrated under reduced pressure, and partitioned between water and ether. The organic layer was washed

with hydrochloric acid (2N), sodium bicarbonate solution, then water, dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The residual green solid was purified by medium pressure column chromatography on silica gel using ethyl acetate:hexane (90:10) as eluant to afford the title compound, 880mg, 57%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) d: 1.37-2.28 (m, 12H), 2.46-2.64 (m, 1H), 3.20 (s, 3H), 3.31 (m, 2H), 4.97 (dd, 2H), 5.08 (dd, 2H), 6.57 (d, 1H), 7.12 (m, 1H), 7.18-7.48 (m, 10H), 8.08 (d, 1H).

### **Preparation 2 (2/28)**

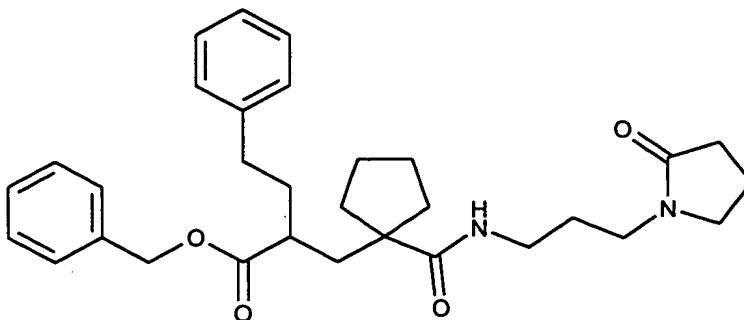
#### **5-Amino-1-benzyl-2(1H)-pyridinone**



A mixture of 1-benzyl-5-nitro-1H-pyridin-2-one (Justus Liebigs Ann. Chem. 484; 1930; 52) (1.0g, 4.35mmol), and granulated tin (3.5g, 29.5mmol) in concentrated hydrochloric acid (14ml) was heated at 90°C for 1.5 hours. The cooled solution was diluted with water, neutralised using sodium carbonate solution, and extracted with ethyl acetate (250ml in total). The combined organic extracts were filtered, dried (MgSO<sub>4</sub>), and evaporated under reduced pressure to give the title compound as a pale green solid, (turned blue with time), 440mg, 51%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 250MHz) δ: 4.12-4.47 (bs, 2H), 5.00 (s, 2H), 6.31 (d, 1H), 6.86 (s, 1H), 7.07 (m, 1H), 7.14-7.42 (m, 5H).

### **Preparation 3 (3/67)**

#### **Benzyl 2-([1-([3-(2-Oxo-1-pyrrolidinyl)propyl]amino)carbonylcyclopentyl]-methyl)-4-phenylbutanoate**

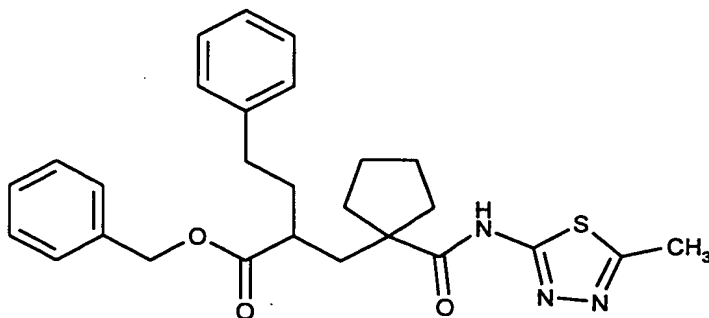


1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.06g, 5.53mmol), 1-hydroxybenzotriazole hydrate (0.60g, 4.44mmol) and 4-methylmorpholine (0.56g, 5.54mmol) were added sequentially to a cooled solution of 1-[2-[(benzyloxy)carbonyl]-4-phenylbutyl]cyclopentane-carboxylic acid (EP 274234) (1.5g,

3.94mmol) in dry dichloromethane (15ml) at room temperature, followed by N-(3-aminopropyl)-2-pyrrolidinone (0.56g, 3.94mmol), and the reaction stirred at room temperature for 18 hours. The mixture was washed with water, 2N hydrochloric acid, saturated aqueous sodium bicarbonate solution, and then dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The residual yellow oil was purified by column chromatography on silica gel using ethyl acetate:pentane (50:50) as the eluant to provide the title compound as a clear gum, 800mg, 40%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) d : 1.37-2.20 (m, 16H), 2.34-2.58 (m, 5H), 2.92-3.46 (m, 6H), 5.07 (d, 1H), 5.18 (d, 1H), 6.98-7.47 (m, 10H).

#### **Preparation 4 (4/70)**

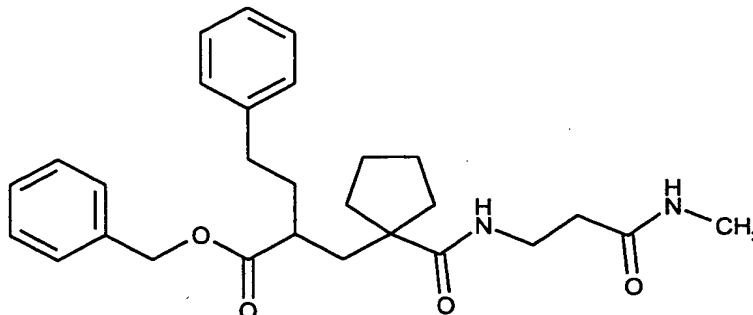
**Benzyl 2-[(1-[(5-methyl-1,3,4-thiadiazol-2-yl)amino]carbonyl)cyclopentyl)methyl]-4-phenylbutanoate**



The title compound was obtained as a clear oil in 74% yield from 1-[2-[(benzyloxy)carbonyl]-4-phenylbutyl]cyclopentane-carboxylic acid (EP 274234) and 2-amino-5-methyl-1,3,4-thiadiazole, following a similar procedure to that described in preparation 5 (5/68); <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) d: 1.58-1.76 (m, 7H), 1.83-1.98 (m, 3H), 2.03 (m, 1H), 2.20 (m, 1H), 2.35 (m, 1H), 2.44 (m, 3H), 2.65 (s, 3H), 5.02 (dd, 2H), 7.00 (d, 2H), 7.15 (m, 1H), 7.19 (m, 2H), 7.35 (m, 5H); LRMS : m/z 478.7 (MH<sup>+</sup>).

**Preparation 5 (5/68)**

Benzyl 2-([1-([3-(methylamino)-3-oxopropyl]amino)carbonyl]cyclopentyl)methyl]-4-phenylbutanoate



5

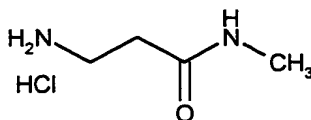
1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (122mg, 0.64mmol), 1-hydroxybenzotriazole hydrate (86mg, 0.64mmol) and 4-methylmorpholine (173μl, 1.59mmol) were added sequentially to a cooled solution of 1-{2-[(benzyloxy)carbonyl]-4-phenylbutyl}cyclopentane-carboxylic acid (EP 274234) (202mg, 0.53mmol) in N,N-dimethylformamide (5ml) at room temperature, followed by the amine hydrochloride from preparation 6 (6/23) (146mg, 1.06mmol), and the reaction stirred at 90°C for 18 hours. The cooled solution was concentrated under reduced pressure and the residue partitioned between water (20ml) and ethyl acetate (100ml). The layers were separated, the organic phase washed with water (3x30ml), brine (25ml) dried (MgSO<sub>4</sub>), and evaporated under reduced pressure to give a clear oil. The crude product was purified by column chromatography on silica gel using dichloromethane:methanol (98:2) as eluant to afford the title compound as a colourless oil, 162mg, 67%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.38-1.53 (m, 2H), 1.53-1.96 (m, 8H), 2.02 (m, 2H), 2.27 (t, 2H), 2.46 (m, 3H), 2.76 (d, 3H), 3.44 (m, 2H), 5.13 (s, 2H), 5.79 (bs, 1H), 6.38 (m, 1H), 7.06 (d, 2H), 7.18 (m, 1H), 7.22 (m, 2H), 7.38 (m, 5H); LRMS : m/z 465.5 (MH<sup>+</sup>).

15

20

**Preparation 6 (6/23)**

3-Amino-N-methylpropanamide hydrochloride



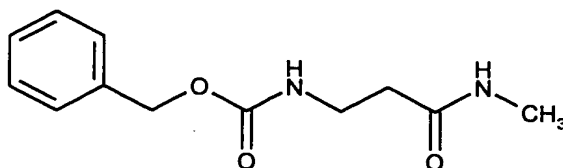
25

A mixture of the benzyl carbamate from preparation 7 (7/13) (7.92g, 33.5mmol) and 5% palladium on charcoal (800mg) in ethanol (300ml) was hydrogenated at 50 psi and room temperature for 4 hours. The reaction mixture was filtered through Arbocel

®, washing through with ethanol, and 1N hydrochloric acid (36.9ml, 36.9mmol) was added to the combined filtrate. This solution was evaporated under reduced pressure and the residue azeotroped with dichloromethane to afford the title compound as a colourless foam, 4.66g, <sup>1</sup>H NMR (DMSO-d<sub>6</sub>, 300MHz) δ: 2.46 (t, 2H), 2.60 (s, 3H),  
 5 2.95 (m, 2H), 7.98-8.16 (m, 2H).

### **Preparation 7 (7/13)**

#### **Benzyl 3-(methylamino)-3-oxopropylcarbamate**



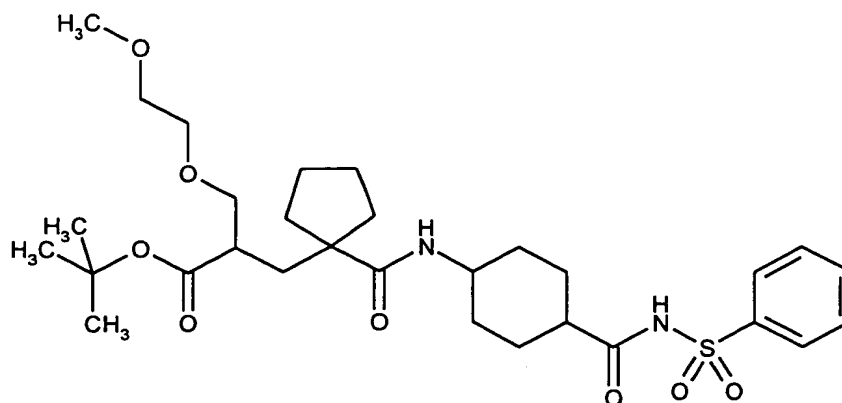
10 A mixture of N-[(benzyloxy)carbonyl]-β-alanine (10g, 44.8mmol), methylamine hydrochloride (3.33g, 49.28mmol), 1-hydroxybenzotriazole hydrate (6.05g, 44.8mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (10.3g, 53.76mmol) and N-methylmorpholine (11.33ml, 103mmol) in dichloromethane (200ml) was stirred at room temperature for 18 hours. The resulting precipitate was  
 15 filtered off to give the desired product as a colourless foam, and the filtrate evaporated under reduced pressure. The residue was purified by column chromatography on silica gel using an elution gradient of ethyl acetate:hexane (90:10 to 100:0) to give additional product, 7.96g, 75% in total; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 2.42 (t, 2H), 2.80 (s, 3H), 3.50 (m, 2H), 5.21 (s, 2H), 5.49 (bs, 1H), 5.63 (bs, 1H),  
 20 7.36 (m, 5H); Anal. Found: C, 60.68; H, 7.00; N, 11.95. C<sub>12</sub>H<sub>16</sub>N<sub>2</sub>O<sub>3</sub> requires C, 61.00; H, 6.83; N, 11.86%.

### **Preparation 8 (8/66)**

#### **Cis-tert-Butyl 3-(2-methoxyethoxy)-2-[(1-[(4-[(phenylsulfonyl)amino]carbonyl]-cyclohexyl)amino]carbonyl)cyclopentyl)methyl]propanoate**

25

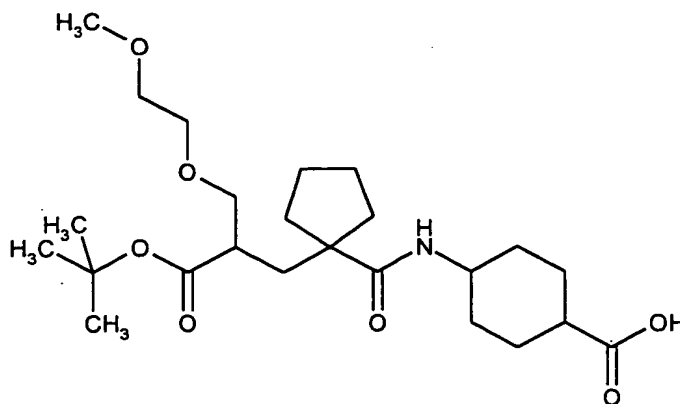




N,N'-Dicyclohexylcarbodiimide (199mg, 0.97mmol), 4-dimethylaminopyridine (118mg, 0.97mmol) and benzenesulphonamide (152mg, 0.97mmol) were added to an ice-cooled solution of the acid from preparation 9 (9/63) (400mg, 0.878mmol) in dichloromethane (12ml) and N,N-dimethylformamide (0.5ml), and the reaction stirred at room temperature for 20 hours. The mixture was concentrated under reduced pressure and the residue suspended in cold ethyl acetate. The resulting insoluble material was filtered off, the filtrate washed with hydrochloric acid (1N), and water, then dried (MgSO<sub>4</sub>) and evaporated under reduced pressure. The crude product was purified by column chromatography on silica gel using an elution gradient of dichloromethane:methanol (95:5 to 90:10) to afford the title compound as a white foam, 480mg, 92%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ: 1.44 (s, 9H), 1.63 (m, 13H), 1.80 (m, 2H), 1.88 (m, 1H), 1.98 (m, 2H), 2.36 (m, 1H), 2.57 (m, 1H), 3.38 (s, 3H), 3.40 (m, 1H), 3.51 (t, 2H), 3.58 (m, 3H), 3.95 (m, 1H), 5.92 (d, 1H), 7.56 (m, 2H), 7.62 (m, 1H), 8.05 (d, 2H), 8.75 (bs, 1H); LRMS : m/z 618 (MNa<sup>+</sup>).

#### **Preparation 9 (9/63)**

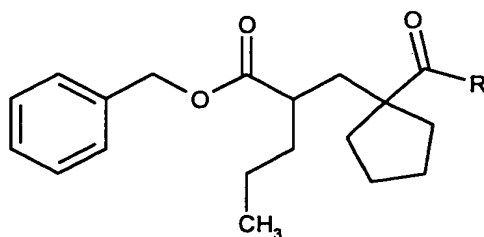
**4-([(1-[3-tert-Butoxy-2-[(2-methoxyethoxy)methyl]-3-oxopropyl]cyclopentyl)-carbonyl]amino)cyclohexanecarboxylic acid**



A mixture of benzyl 4-[[1-(3-tert-butoxy-2-[(2-methoxyethoxy)methyl]-3-oxopropyl)cyclopentyl]carbonylamino]cyclohexanecarboxylate (EP 274234), and 10% palladium on charcoal (250mg) in water (10ml) and ethanol (50ml) was hydrogenated at 50 psi and room temperature for 18 hours. The reaction mixture was filtered through Solkafloc®, the filtrate concentrated under reduced pressure and the residue azeotroped with toluene (3x) and then dichloromethane (3x), to give the title compound, 2.0g, 96%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) d: 1.48 (s, 9H), 1.53-1.84 (m, 14H), 1.94-2.10 (m, 5H), 2.60 (m, 2H), 3.40 (s, 3H), 3.41-3.63 (m, 5H), 3.96 (m, 1H), 5.90 (bd, 1H).

#### Preparation 10 (10/53)

The following compound:



where:

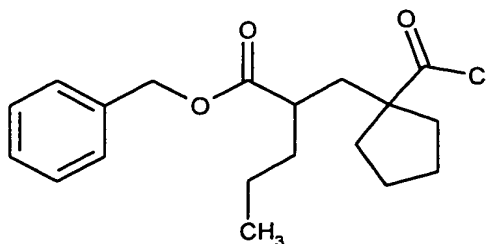
Prep	R	Yield (%)	Data
10 (10/53) <sup>1</sup>		90	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 300MHz) δ: 0.84 (t, 3H), 1.24 (m, 2H), 1.40-1.76 (m, 7H), 1.84 (dd, 1H), 1.98 (m, 1H), 2.19 (dd, 1H), 2.28 (m, 1H), 2.56 (m, 1H), 3.98 (s, 2H), 4.99 (dd, 2H), 6.98 (d, 1H), 7.19-7.42 (m, 15H).

<sup>1</sup> = dichloromethane used as the column eluant

was prepared from the acid chloride from preparation 11 (11/3) and the appropriate amine, following a similar procedure to that described in preparation 12 (12/52).

#### Preparation 11 (11/3)

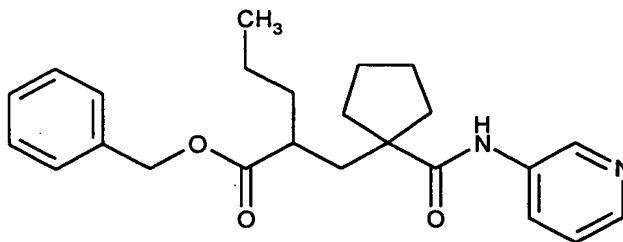
Benzyl 2-[[1-(chlorocarbonyl)cyclopentyl]methyl]pentanoate



Oxalyl chloride (1.15ml, 13.2mmol) was added to an ice-cooled solution of 1-{2-[(benzyloxy)carbonyl]pentyl}cyclopentanecarboxylic acid (EP 274234) (2.0g, 6.3mmol) in dry dichloromethane (20ml), and the solution stirred at room temperature for 2 hours. The reaction mixture was concentrated under reduced pressure and the residue azeotroped with dichloromethane (3x), to give the title compound as a golden oil, 2.1g;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 300MHz)  $\delta$ : 0.88 (t, 3H), 1.28 (m, 2H), 1.43 (m, 2H), 1.63 (m, 6H), 2.00 (m, 1H), 2.08-2.35 (m, 3H), 2.44 (m, 1H), 5.15 (s, 2H), 7.28 (m, 5H).

#### **Preparation 12 (12/52)**

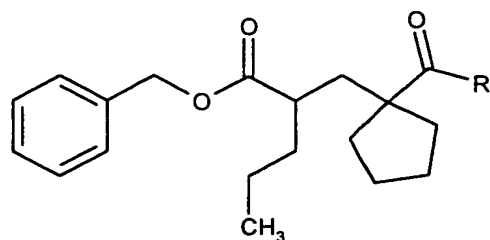
**Benzyl 2-({1-[3-(pyridinylamino)carbonyl]cyclopentyl}methyl)pentanoate**



Triethylamine (0.11ml, 0.78mmol) was added to a mixture of the acid chloride from preparation 11 (11/3) (200mg, 0.60mmol) and 2-aminopyridine (61mg, 0.65mmol) in dichloromethane (3ml), and the reaction stirred at room temperature for 16 hours. The mixture was evaporated under reduced pressure, the residue partitioned between sodium bicarbonate solution (5ml) and ethyl acetate (20ml), and the layers separated. The organic phase was dried ( $\text{MgSO}_4$ ), and evaporated under reduced pressure to give a gum. The crude product was purified by column chromatography on silica gel using ethyl acetate as eluant, to afford the title compound, 130mg;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz)  $\delta$ : 0.82 (t, 3H), 1.21 (m, 3H), 1.40 (m, 1H), 1.43-1.72 (m, 6H), 1.81 (d, 1H), 1.98 (m, 1H), 2.18 (m, 1H), 2.24 (m, 1H), 2.46 (m, 1H), 4.98 (m, 2H), 7.20-7.38 (m, 6H), 7.42 (s, 1H), 8.06 (d, 1H), 8.35 (d, 1H), 8.56 (s, 1H).

#### **Preparation 13 (13/56)**

The following compound:



where:

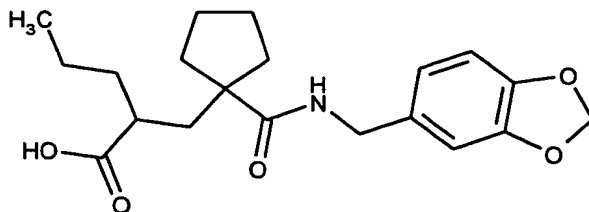
Prep	R	Yield (%)	Data
13 (13/56) <sup>2</sup>		53	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 300MHz) δ: 0.84 (t, 3H), 1.25 (m, 2H), 1.27-1.99 (m, 10H), 2.07-2.30 (m, 2H), 2.47 (m, 1H), 4.99 (s, 2H), 5.10 (dd, 2H), 6.59 (d, 1H), 7.15 (d, 1H), 7.34 (m, 11H), 8.10 (s, 1H).

5 2 = N-methylmorpholine was used as the base

was prepared from the acid chloride from preparation 11 (11/3) and the appropriate amine, following a similar procedure to that described in preparation 12 (12/52).

10 **Preparation 14 (14/ex 1)**

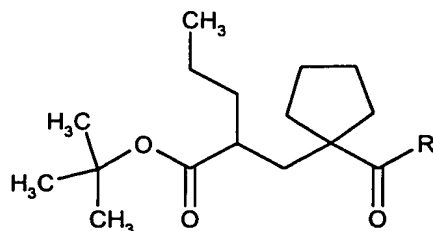
2-({1-[(1,3-Benzodioxol-5-ylamino)carbonyl]cyclopentyl}methyl)pentanoic acid



Trifluoroacetic acid (5ml) was added to a solution of the tert-butyl ester from preparation 15 (15/34) (130mg, 0.31mmol) in dichloromethane (5ml), and the solution stirred at room temperature for 4 hours. The reaction mixture was concentrated under reduced pressure and the residue azeotroped with toluene and dichloromethane to afford the title compound as a clear oil, 112 mg, <sup>1</sup>H NMR (CDCl<sub>3</sub>, 400MHz) δ 0.83 (t, 3H), 1.22-1.40 (m, 3H), 1.50-1.72 (m, 8H), 1.95 (m, 1H), 2.10 (m, 2H), 2.19 (m, 1H), 4.30 (m, 2H), 5.93 (s, 2H), 5.99 (bs, 1H), 6.74 (m, 3H); LRMS: m/z 380 (MH<sup>+</sup>).

**Preparation 15 (15/34)**

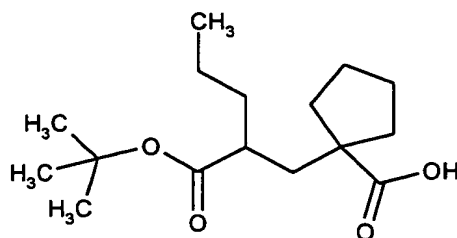
The following compound:



5 where

Prep	R	Starting amine	Yield (%)	Data
15 (15/34)		Piperonylamine	88	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) δ: 0.85 (t, 3H), 1.26 (m, 4H), 1.42 (s, 9H), 1.46 (m, 2H), 1.59-1.75 (m, 5H), 1.95 (m, 2H), 2.06 (m, 1H), 2.22 (m, 1H), 4.26 (dd, 1H), 4.39 (dd, 1H), 5.95 (m, 3H), 6.78 (m, 3H). LRMS : m/z 418.3 (MH <sup>+</sup> )

10 was prepared from the acid from preparation 16 (16/1) and the appropriate amine compound, following a similar procedure to that described in preparation 17 (17/33).

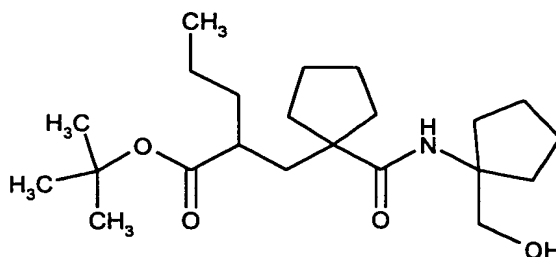
**Preparation 16 (16/1)****1-[2-(tert-Butoxycarbonyl)-4-pentyl]-cyclopentane carboxylic acid**

15 A mixture of 1-[2-(tert-butoxycarbonyl)-4-pentenyl]-cyclopentane carboxylic acid (EP 274234) (23g, 81.5mmol) and 10% palladium on charcoal (2g) in dry ethanol (200ml) was hydrogenated at 30psi and room temperature for 18 hours. The reaction mixture was filtered through Arbocel®, and the filtrate evaporated under reduced pressure to give a yellow oil. The crude product was purified by column chromatography on silica

gel, using ethyl acetate:pentane (40:60) as the eluant, to provide the desired product as a clear oil, 21g, 91%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 0.86 (t, 3H), 1.22-1.58 (m, 15H), 1.64 (m, 4H), 1.78 (dd, 1H), 2.00-2.18 (m, 3H), 2.24 (m, 1H); LRMS : m/z 283 (M-H) $^-$

5 **Preparation 17 (17/33)**

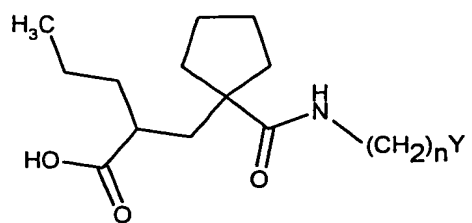
**tert-Butyl 2-([1-([1-(hydroxymethyl)cyclopentyl]amino)carbonyl]-cyclopentyl)methyl]pentanoate**



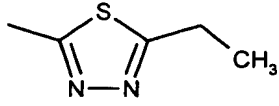
1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (41mg, 0.21mmol), 1-  
 10 hydroxybenzotriazole hydrate (27mg, 0.2mmol), N-methylmorpholine (35 $\mu$ l, 0.31mmol) and finally 1-amino-1-cyclopentanemethanol (25mg, 0.22mmol) were added to a solution of the acid from preparation 16 (16/1) (150mg, 0.53mmol) in N,N-dimethylformamide (3ml), and the reaction stirred at 90°C for 18 hours. The cooled solution was diluted with ethyl acetate (90ml), washed with water (3x25ml), and brine  
 15 (25ml), then dried ( $\text{MgSO}_4$ ) and evaporated under reduced pressure. The crude product was purified by chromatography on silica gel, using ethyl acetate:pentane (30:70) as the eluant to afford the title compound, 38mg, 57%;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 400MHz) d: 0.88 (t, 3H), 1.29 (m, 3H), 1.41-1.78 (m, 26H), 1.78-1.98 (m, 4H), 2.04 (m, 1H), 2.26 (m, 1H), 3.59 (dd, 1H), 3.70 (dd, 1H), 4.80 (t, 1H), 5.81 (s, 1H); LRMS :  
 20 m/z 380 (MH $^+$ ).

**Preparation 18 (18/ex.4)**

A compound of the formula shown below was prepared from the corresponding *tert*-  
 25 butyl ester following a similar procedure to that described in Preparation 14 (14/ex.1).



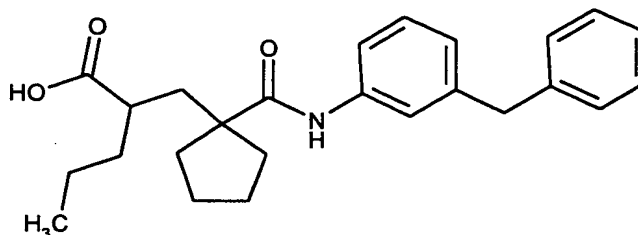
(Ic)

Ex	N	R	Yield	Data
18 (18/ ex.4 ) <sup>3</sup>	0		86	<sup>1</sup> H NMR (CDCl <sub>3</sub> , 400MHz) δ: 0.92 (t, 3H), 1.35 (t, 3H), 1.25-1.80 (m, 11H), 2.20-2.50 (m, 4H), 2.95 (q, 2H), 12.10 (bs, 1H). LRMS : m/z 339.8 (MH <sup>+</sup> ) Anal. Found: C, 56.46; H, 7.46; N, 12.36. C <sub>16</sub> H <sub>25</sub> N <sub>3</sub> O <sub>3</sub> S requires C, 56.62; H, 7.44; N, 12.37%.

3 = recrystallised from ether

5 **Preparation 19 (19/ex.21)**

2-({1-[3-Benzylanilino)carbonyl]cyclopentyl)methyl}pentanoic acid



A mixture of the benzyl ester from preparation 10 (10/53) (1.3mg, 2.47mmol) and 5% palladium on charcoal (130mg) in water (10ml) and ethanol (40ml) was hydrogenated at 30 psi and room temperature for 2 hours. The reaction mixture was filtered through Arbocel®, the filtrate concentrated under reduced pressure, and the residue triturated with dichloromethane. The residual gum was triturated with ether, then hexane, and dried at 50°C, to give the title compound as a solid, 0.79g, 81%; <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300MHz) δ: 0.95 (t, 3H), 1.24-1.51 (m, 3H), 1.58-1.80 (m, 7H), 1.88 (dd, 1H), 2.15 (m, 2H), 2.24 (m, 1H), 2.48 (m, 1H), 4.00 (s, 2H), 6.98 (d, 1H), 7.24 (m, 6H), 7.40 (m, 3H); Anal. Found: C, 75.48; H, 7.76; N, 3.59. C<sub>25</sub>H<sub>31</sub>NO<sub>3</sub>·0.25H<sub>2</sub>O requires C, 75.44; H, 7.98; N, 3.51%.



5 All publications mentioned in the above specification are herein incorporated by  
reference. Various modifications and variations of the described methods and  
system of the present invention will be apparent to those skilled in the art without  
departing from the scope and spirit of the present invention. Although the present  
invention has been described in connection with specific preferred embodiments, it  
10 should be understood that the invention as claimed should not be unduly limited to  
such specific embodiments. Indeed, various modifications of the described modes  
for carrying out the invention which are obvious to those skilled in biochemistry and  
biotechnology or related fields are intended to be within the scope of the following  
claims.

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## 25 ABBREVIATIONS

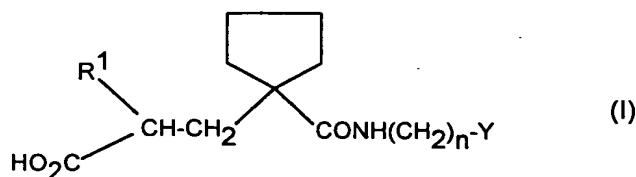
	cAMP	=	cyclic adenosine-3',5'-monophosphate
	cGMP	=	cyclic guanosine-3',5'-monophosphate
30	P <sub>cGMP</sub>	=	potentiator of cGMP
	NEP	=	neutral endopeptidase
	NEPi	=	inhibitor of NEP (also known as I:NEP)
35	VIP	=	vasoactive intestinal peptide
	PDE	=	phosphodiesterase

	PDEn	=	PDE family (e.g. PDE1, PDE2 etc.)
	PDE <sub>cGMP</sub>	=	cGMP hydrolysing PDE
	PDEi	=	inhibitor of a PDE (also known as I:PDE)
5	NPY	=	neuropeptide Y
	I:NPY	=	inhibitor of NPY
	kDa	=	kilodalton
	bp	=	base pair
10	kb	=	kilobase pair

# Claims

1 The use of a NEPi compound for the treatment of MED.

5 2 Use according to claim 1 wherein the NEPi is a compound of formula I (or a pharmaceutically acceptable salt, solvate or prodrug thereof):



wherein

10  $R^1$  is  $C_{1-6}$ alkyl which may be substituted by one or more substituents, which may be the same or different, selected from the list: halo, hydroxy,  $C_{1-6}$  alkoxy,  $C_{2-6}$  hydroxyalkoxy,  $C_{1-6}$ alkoxy( $C_{1-6}$ alkoxy),  $C_{3-7}$ cycloalkyl,  $C_{3-7}$ cycloalkenyl, aryl, aryloxy, ( $C_{1-4}$ alkoxy)aryloxy, heterocyclyl, heterocyclyloxy,  $-NR^2R^3$ ,  $-NR^4COR^5$ ,  $-NR^4SO_2R^5$ ,  $-CONR^2R^3$ ,  $-S(O)_pR^6$ ,  $-COR^7$  and  $-CO_2(C_{1-4}$ alkyl); or  $R^1$  is  
15  $C_{3-7}$ cycloalkyl, aryl or heterocyclyl, each of which may be substituted by one or more substituents from said list, which substituents may be the same or different, which list further includes  $C_{1-6}$ alkyl; or  $R^1$  is  $C_{1-6}$  alkoxy,  $-NR^2R^3$  or  $-NR^4SO_2R^5$ ;

wherein

20  $R^2$  and  $R^3$  are each independently H,  $C_{1-4}$ alkyl,  $C_{3-7}$ cycloalkyl (optionally substituted by hydroxy or  $C_{1-4}$ alkoxy), aryl, ( $C_{1-4}$ alkyl)aryl,  $C_{1-6}$ alkoxyaryl or heterocyclyl; or  $R^2$  and  $R^3$  together with the nitrogen to which they are attached form a pyrrolidinyl, piperidino, morpholino, piperazinyl or  $N-(C_{1-4}$  alkyl)piperazinyl group;

25  $R^4$  is H or  $C_{1-4}$ alkyl;

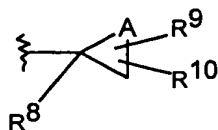
$R^5$  is  $C_{1-4}$ alkyl,  $CF_3$ , aryl, ( $C_{1-4}$  alkyl)aryl, ( $C_{1-4}$ alkoxy)aryl, heterocyclyl,  $C_{1-4}$ alkoxy or  $-NR^2R^3$  wherein  $R^2$  and  $R^3$  are as previously defined;

$R^6$  is  $C_{1-4}$ alkyl, aryl, heterocyclyl or  $NR^2R^3$  wherein  $R^2$  and  $R^3$  are as previously defined; and

$R^7$  is  $C_{1-4}$ alkyl,  $C_{3-7}$ cycloalkyl, aryl or heterocyclyl; n is 0, 1 or 2; p is 0, 1, 2 or 3;

the  $-(CH_2)_n-$  linkage is optionally substituted by  $C_{1-4}$ alkyl,  $C_{1-4}$ alkyl substituted with one or more fluoro groups or phenyl,  $C_{1-4}$ alkoxy, hydroxy, hydroxy( $C_{1-3}$ alkyl),  $C_{3-7}$ cycloalkyl, aryl or heterocyclyl;

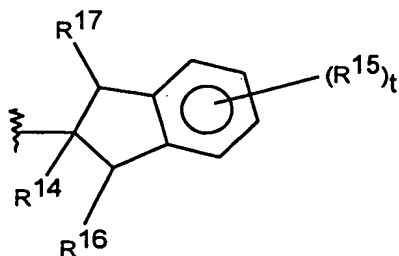
Y is the group



wherein A is  $-(CH_2)_q-$  where q is 1, 2, 3 or 4 to complete a 3 to 7 membered carbocyclic ring which may be saturated or unsaturated;  $R^8$  is H,  $C_{1-6}$ alkyl,  $-CH_2OH$ , phenyl, phenyl( $C_{1-4}$ alkyl) or  $CONR^{11}R^{12}$ ;  $R^9$  and  $R^{10}$  are each independently H,  $-CH_2OH$ ,  $-C(O)NR^{11}R^{12}$ ,  $C_{1-6}$ alkyl, phenyl optionally substituted by  $C_{1-4}$ alkyl, or phenyl( $C_{1-4}$ alkyl) wherein the phenyl group is optionally substituted by  $C_{1-4}$ alkyl, or  $R^9$  and  $R^{10}$  together form a dioxolane;  $R^{11}$  and  $R^{12}$  which may be the same or different are H,  $C_{1-4}$ alkyl,  $R^{13}$  or  $S(O)_rR^{13}$ , where r is 0, 1 or 2 and  $R^{13}$  is phenyl optionally substituted by  $C_{1-4}$ alkyl or phenyl( $C_{1-4}$ alkyl) wherein the phenyl is optionally substituted by  $C_{1-4}$ alkyl; or

Y is the group,  $-C(O)NR^{11}R^{12}$  wherein  $R^{11}$  and  $R^{12}$  are as previously defined except that  $R^{11}$  and  $R^{12}$  are not both H; or

Y is the group,

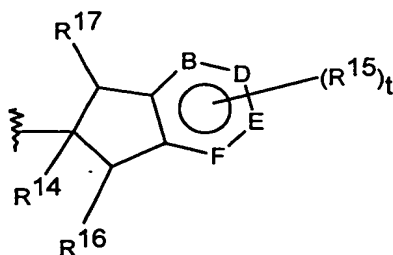


wherein  $R^{14}$  is H,  $CH_2OH$ , or  $C(O)NR^{11}R^{12}$  wherein  $R^{11}$  and  $R^{12}$  are as previously defined; when present  $R^{15}$ , which may be the same or different to any other  $R^{15}$ , is OH,  $C_{1-4}$ alkyl,  $C_{1-4}$ alkoxy, halo or  $CF_3$ ; t

is 0, 1, 2, 3 or 4; and  $R^{16}$  and  $R^{17}$  are independently H or  $C_{1-4}$  alkyl;

or

Y is the group



wherein one or two of B, D, E or F is a nitrogen, the others being carbon; and

$R^{14}$  to  $R^{17}$  and  $t$  are as previously defined; or

Y is an optionally substituted 5-7 membered heterocyclic ring, which may be saturated, unsaturated or aromatic and contains a nitrogen, oxygen or sulphur and optionally one, two or three further nitrogen atoms in the ring and which may be optionally benzofused and optionally substituted by:

$C_{1-6}$  alkoxy; hydroxy; oxo; amino; mono or di- $(C_{1-4}$ alkyl)amino;

$C_{1-4}$ alkanoylamino; or

$C_{1-6}$ alkyl which may be substituted by one or more groups, which may

be the same or different, selected from the list:  $C_{1-6}$ alkoxy,

$C_{1-6}$ haloalkoxy,  $C_{1-6}$ alkylthio, halogen,  $C_{3-7}$ cycloalkyl,

heterocyclyl or phenyl; or

$C_{3-7}$ cycloalkyl, aryl or heterocyclyl, each of which may be substituted

by one or more groups, which may be the same or different,

selected from the list:  $C_{1-6}$ alkyl,  $C_{1-6}$ alkoxy,  $C_{1-6}$ haloalkoxy,

$C_{1-6}$ alkylthio, halogen,  $C_{3-7}$ cycloalkyl, heterocyclyl or phenyl;

wherein when there is an oxo substitution on the heterocyclic ring, the

ring only contains one or two nitrogen atoms and the oxo

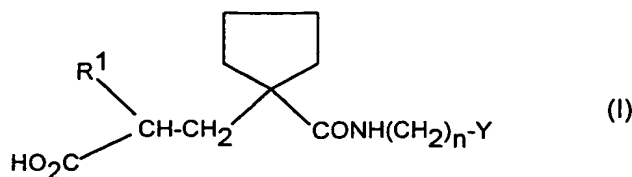
substitution is adjacent a nitrogen atom in the ring; or

Y is  $-NR^{18}S(O)_uR^{19}$ , wherein  $R^{18}$  is H or  $C_{1-4}$ alkyl;  $R^{19}$  is aryl, aryl $C_{1-4}$ alkyl or heterocyclyl; and

$u$  is 0, 1, 2 or 3.

Use according to claim 1 wherein the NEPi is compound of formula (I), or a pharmaceutically acceptable salt, solvate or prodrug thereof:





wherein

$R^1$  is  $C_{1-6}$ alkyl which may be substituted by one or more substituents, which may be the same or different, selected from the list: halo, hydroxy,  $C_{1-6}$  alkoxy,  $C_{2-6}$  hydroxyalkoxy,  $C_{1-6}$ alkoxy( $C_{1-6}$ alkoxy),  $C_{3-7}$ cycloalkyl,  $C_{3-7}$ cycloalkenyl, aryl, aryloxy, ( $C_{1-4}$ alkoxy)aryloxy, heterocyclyl, heterocycliloxy,  $-NR^2R^3$ ,  $-NR^4COR^5$ ,  $-NR^4SO_2R^5$ ,  $-CONR^2R^3$ ,  $-S(O)_pR^6$ ,  $-COR^7$  and  $-CO_2(C_{1-4}alkyl)$ ; or  $R^1$  is  $C_{3-7}$ cycloalkyl, aryl or heterocyclyl, each of which may be substituted by one or more substituents from said list, which substituents may be the same or different, which list further includes  $C_{1-6}$ alkyl; or  $R^1$  is  $C_{1-6}$  alkoxy,  $-NR^2R^3$  or  $-NR^4SO_2R^5$ ;

wherein

$R^2$  and  $R^3$  are each independently H,  $C_{1-4}$ alkyl,  $C_{3-7}$ cycloalkyl (optionally substituted by hydroxy or  $C_{1-4}$ alkoxy), aryl, ( $C_{1-4}$ alkyl)aryl,  $C_{1-6}$ alkoxyaryl or heterocyclyl; or  $R^2$  and  $R^3$  together with the nitrogen to which they are attached form a pyrrolidinyl, piperidino, morpholino, piperazinyl or  $N-(C_{1-4}alkyl)$ piperazinyl group;

$R^4$  is H or  $C_{1-4}$ alkyl;

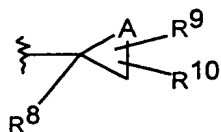
$R^5$  is  $C_{1-4}$ alkyl,  $CF_3$ , aryl, ( $C_{1-4}alkyl$ )aryl, ( $C_{1-4}$ alkoxy)aryl, heterocyclyl,  $C_{1-4}$ alkoxy or  $-NR^2R^3$  wherein  $R^2$  and  $R^3$  are as previously defined;

$R^6$  is  $C_{1-4}$ alkyl, aryl, heterocyclyl or  $NR^2R^3$  wherein  $R^2$  and  $R^3$  are as previously defined; and

$R^7$  is  $C_{1-4}$ alkyl,  $C_{3-7}$ cycloalkyl, aryl or heterocyclyl;  $n$  is 0, 1 or 2;  $p$  is 0, 1, 2 or 3;

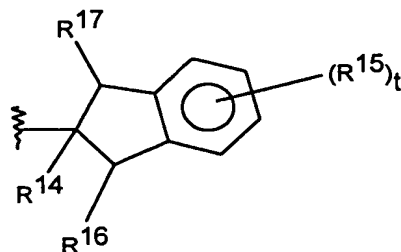
the  $-(CH_2)_n-$  linkage is optionally substituted by  $C_{1-4}$ alkyl,  $C_{1-4}$ alkyl substituted with one or more fluoro groups or phenyl,  $C_{1-4}$ alkoxy, hydroxy, hydroxy( $C_{1-3}$ alkyl),  $C_{3-7}$ cycloalkyl, aryl or heterocyclyl;

Y is the group



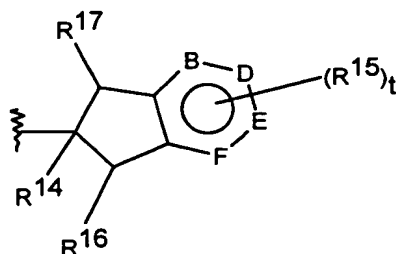
wherein A is  $-(CH_2)_q-$  where q is 1, 2, 3 or 4 to complete a 3 to 7 membered carbocyclic ring which may be saturated or unsaturated;  
 $R^8$  is H,  $C_{1-6}$ alkyl,  $-CH_2OH$ , phenyl, phenyl( $C_{1-4}$ alkyl) or  
 $CONR^{11}R^{12}$ ;  $R^9$  and  $R^{10}$  are each independently H,  $-CH_2OH$ ,  
 $-C(O)NR^{11}R^{12}$ ,  $C_{1-6}$ alkyl, phenyl optionally substituted by  $C_{1-4}$ alkyl,  
or phenyl( $C_{1-4}$ alkyl) wherein the phenyl group is optionally substituted  
by  $C_{1-4}$ alkyl, or  $R^9$  and  $R^{10}$  together form a dioxolane;  $R^{11}$  and  $R^{12}$   
which may be the same or different are H,  $C_{1-4}$ alkyl,  $R^{13}$  or  
 $S(O)_rR^{13}$ , where r is 0, 1 or 2 and  $R^{13}$  is phenyl optionally substituted  
by  $C_{1-4}$ alkyl or phenyl $C_{1-4}$ alkyl wherein the phenyl is optionally  
substituted by  $C_{1-4}$ alkyl; or

Y is the group,



wherein  $R^{14}$  is  $C(O)NR^{11}R^{12}$  wherein  $R^{11}$  and  $R^{12}$  are as previously defined; when present  $R^{15}$ , which may be the same or different to any other  $R^{15}$ , is OH,  $C_{1-4}$ alkyl,  $C_{1-4}$ alkoxy, halo or  $CF_3$ ; t is 0, 1, 2, 3 or 4; and  $R^{16}$  and  $R^{17}$  are independently H or  $C_{1-4}$  alkyl; or

Y is the group



wherein one or two of B, D, E or F is a nitrogen, the others being carbon; and R<sup>14</sup> to R<sup>17</sup> and t are as previously defined; or

Y is an optionally substituted 5-7 membered heterocyclic ring, which may be saturated, unsaturated or aromatic and contains a nitrogen, oxygen or sulphur and optionally one, two or three further nitrogen atoms in the ring and which may be optionally benzofused and optionally substituted by:

C<sub>1-6</sub> alkoxy; hydroxy; oxo; amino; mono or di-(C<sub>1-4</sub>alkyl)amino;

C<sub>1-4</sub>alkanoylamino; or

C<sub>1-6</sub>alkyl which may be substituted by one or more groups, which may be the same or different, selected from the list: C<sub>1-6</sub>alkoxy, C<sub>1-6</sub>haloalkoxy, C<sub>1-6</sub>alkylthio, halogen, C<sub>3-7</sub>cycloalkyl, heterocyclyl or phenyl; or

C<sub>3-7</sub>cycloalkyl, aryl or heterocyclyl, each of which may be substituted by one or more groups, which may be the same or different, selected from the list: C<sub>1-6</sub>alkyl, C<sub>1-6</sub>alkoxy, C<sub>1-6</sub>haloalkoxy, C<sub>1-6</sub>alkylthio, halogen, C<sub>3-7</sub>cycloalkyl, heterocyclyl or phenyl;

wherein when there is an oxo substitution on the heterocyclic ring, the ring only contains one or two nitrogen atoms and the oxo substitution is adjacent a nitrogen atom in the ring; or

Y is -NR<sup>18</sup>S(O)<sub>u</sub>R<sup>19</sup>, wherein R<sup>18</sup> is H or C<sub>1-4</sub>alkyl; R<sup>19</sup> is aryl, arylC<sub>1-4</sub>alkyl or heterocyclyl; and

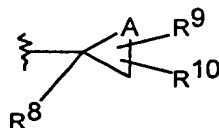
u is 0, 1, 2 or 3.

4. Use according to claim 1 wherein the NEPi is a compound as defined in claims 2 or 3 wherein R<sup>1</sup> is C<sub>1-6</sub>alkyl, C<sub>1-6</sub>alkoxy or C<sub>1-6</sub>alkyl substituted with aryl.

5 Use of a compound as defined in claim 4 wherein R<sup>1</sup> is C<sub>1-6</sub>alkyl, C<sub>1-6</sub>alkoxy or C<sub>1-6</sub>alkoxy(C<sub>1-3</sub>)alkyl.

6 Use of a compound as defined in claim 5 wherein R<sup>1</sup> is C<sub>1-4</sub>alkyl.

- 7 Use of a compound as defined in any one of the preceding claims wherein when Y is the group



- and the carbocyclic ring is fully saturated, then one of R<sup>9</sup> or R<sup>10</sup> is -CH<sub>2</sub>OH,  
 5 -C(O)NR<sup>11</sup>R<sup>12</sup>, C<sub>1-6</sub>alkyl, phenyl optionally substituted by C<sub>1-4</sub>alkyl or phenyl(C<sub>1-4</sub>alkyl) wherein the phenyl group is optionally substituted by C<sub>1-4</sub>alkyl.
- 8 Use of a compound as defined in claim 7 wherein the carbocyclic ring is 5, 6  
 10 or 7 membered wherein one of R<sup>9</sup> or R<sup>10</sup>, -C(O)NR<sup>11</sup>R<sup>12</sup>, with the other being C<sub>1-6</sub>alkyl, phenyl optionally substituted by C<sub>1-4</sub>alkyl or phenyl(C<sub>1-4</sub>alkyl) wherein the phenyl group is optionally substituted by C<sub>1-4</sub>alkyl.
- 9 Use of a compound as defined in claim 8 wherein R<sup>9</sup> and R<sup>10</sup> are attached to  
 15 adjacent carbon atoms in the ring.
- 10 Use of a compound as defined in any one of the preceding claims wherein R<sup>8</sup> is CH<sub>2</sub>OH.
- 20 11 Use of a compound as defined in any of claims 2 to 6 wherein when Y is the group -NR<sup>18</sup>S(O)<sub>u</sub>R<sup>19</sup>, then R<sup>18</sup> is H.
- 12 Use of a compound as defined in any of claims 2 to 6 or 11 wherein R<sup>19</sup> is benzyl or phenyl.  
 25
- 13 Use of a compound as defined in any of claims 2 to 6, 11 or 12 wherein u is 2.
- 14 Use of a compound as defined in claim 2 wherein Y is an optionally  
 30 substituted 5-7 membered heterocyclic ring, which may be saturated, unsaturated or aromatic and contains a nitrogen, oxygen or sulphur and optionally one, two or three further nitrogen atoms in the ring and which may

be optionally benzofused and optionally substituted by one or more of C<sub>1-6</sub> alkyl, phenyl, phenylC<sub>1-4</sub>alkyl, C<sub>1-6</sub> alkoxy, hydroxy, oxo, amino, mono or di-(C<sub>1-4</sub>alkyl)amino or C<sub>1-4</sub> alkanoylamino; with the proviso that when there is an oxo substitution, the ring only contains one or two nitrogen atoms and the oxo substitution is adjacent a nitrogen atom in the ring.

5

15 Use of a compound as defined in claim 14 wherein the heterocyclic ring is a heteroaromatic ring.

10 16 Use of a compound as defined in claim 15 wherein the heteroaromatic ring is selected from pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, pyrazolyl, triazolyl, tetrazolyl, oxadiazole, thiazole, thiaiazole, oxazolyl, isoxazolyl, indolyl, isoindolyl, quinolyl, pyridone, quinoxalyl, and quinazolinyl each being optionally substituted as defined in claim 1.

15

17 Use of a compound as defined in claim 16 wherein the heteroaromatic ring is selected from oxadiazole, pyridone and thiadiazole.

20

18 Use of a compound as defined in claim 17 wherein the heteroaromatic ring is selected from 1,2,5 oxadiazole, 1,3,4 oxadiazole, 2-pyridone and 1,3,4 thiadiazole.

25

19 Use of a compound as defined in any of claims 14 to 18 wherein the heterocyclic ring is substituted by one or more C<sub>1-6</sub>alkyl, phenyl or phenylC<sub>1-4</sub>alkyl.

20 Use of a compound as defined in claim 19 wherein the substitution is C<sub>1-4</sub>alkyl or benzyl.

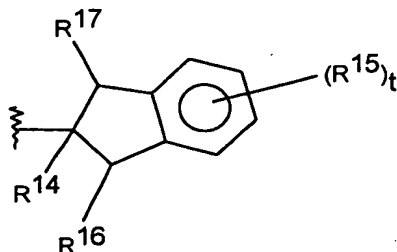
30

21 Use of a compound as defined in claims 17 to 20 wherein when Y is a pyridone, said pyridone is *N*-substituted (preferably with benzyl or C<sub>1-4</sub>alkyl).

22 Use of a compound as defined in claim 14 wherein Y is a lactam linked at the nitrogen.

35

- 23 Use of a compound as defined in claim 2 and claims dependent thereon wherein Y is



- 5 wherein R<sup>14</sup> is H, CH<sub>2</sub>OH or C(O)NR<sup>11</sup>R<sup>12</sup>, R<sup>15</sup> is one or more of H, OH, C<sub>1-4</sub>alkyl, C<sub>1-4</sub>alkoxy, halo or CF<sub>3</sub>; and R<sup>16</sup> and R<sup>17</sup> are independently H or C<sub>1-4</sub>alkyl.
- 24 Use of a compound as defined in claim 23 wherein R<sup>14</sup> is CH<sub>2</sub>OH or  
10 C(O)NR<sup>11</sup>R<sup>12</sup>.
- 25 Use of a compound as defined in claim 24 wherein R<sup>14</sup> is C(O)NR<sup>11</sup>R<sup>12</sup>.
- 26 Use of a compound as defined in claims 3 and claims 23 to 25 wherein R<sup>16</sup>  
15 and R<sup>17</sup> are H.
- 27 Use of a compound as defined in claims 3 and claims 22 to 25 wherein t is 0.
- 28 Use of a compound according to any one of the preceding claims selected  
20 from the group consisting of:
- 2-[(1-[(1-benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl)-cyclopentyl)methyl]-4-methoxybutanoic acid (Example 35),
- 2-[[1-([3-(2-oxo-1-pyrrolidinyl)propyl]amino)carbonylcyclopentyl]-methyl]-4-phenylbutanoic acid (Example 40),
- 25 (+)-2-[[1-([2-(hydroxymethyl)-2,3-dihydro-1H-inden-2-yl]amino)carbonyl]cyclopentyl]-methyl]-4-phenylbutanoic acid (Example 44),
- 2-[(1-[(5-methyl-1,3,4-thiadiazol-2-yl)amino]carbonyl)cyclopentyl]-methyl]-4-phenylbutanoic acid (Example 43),

cis-3-(2-methoxyethoxy)-2-[(1-[(4-[(phenylsulfonyl)amino]carbonyl)-cyclohexyl]amino]carbonyl)cyclopentyl)methyl]propanoic acid (Example 38),

(+)-2-[(1-[(2-(hydroxymethyl)-2,3-dihydro-1*H*-inden-2-yl)amino]carbonyl)-cyclopentyl)methyl]pentanoic acid (Example 31),

(+)-2-[(1-[(5-ethyl-1,3,4-thiadiazol-2-yl)amino]carbonyl)cyclopentyl)-methyl]pentanoic acid (Example 30),

2-[(1-[(3-benzylanilino)carbonyl)cyclopentyl)methyl]pentanoic acid (Example 21),

2-[(1-[(1-benzyl-6-oxo-1,6-dihydro-3-pyridinyl)amino]carbonyl)cyclopentyl)-methyl]-pentanoic acid (Example 22), and

2-[(1-[(1*R*,3*S*,4*R*)-4-(aminocarbonyl)-3-butylcyclohexyl]amino]carbonyl)-cyclopentyl)methyl]pentanoic acid (Example 9).

29 Use of a compound as defined in any one of the preceding claims wherein the chiral carbon attached to R<sup>1</sup> is preferably the R-enantiomer.

30 Use of a NEPi compound according to any one of claims 1 to 29 for the treatment of MED wherein the medicament is administered systemically.

31 Use according to claim 30 wherein the medicament is administered orally.

32 Use of a pharmaceutical formulation including a NEPi compound together with a pharmaceutically acceptable excipient for the treatment of MED.

33. Use according to claim 32 wherein the NEPi compound is as defined in any of claims 2 to 29.

34. Use of a NEPi and a PDE5i for the treatment of MED.

35. Use according to claim 34 wherein the NEPi is as defined in any of claims 1 to 29 and wherein the PDE5i is selected from:

5-[2-ethoxy-5-(4-methyl-1-piperazinylsulphonyl)phenyl]-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one (sildenafil) also known as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)-4-ethoxyphenyl]sulphonyl]-4-methylpiperazine;

5

5-(2-ethoxy-5-morpholinoacetylphenyl)-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-n-propoxyphenyl]-2-(pyridin-2-yl)methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

10

3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-(2-methoxyethoxy)pyridin-3-yl]-2-(pyridin-2-yl)methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

15

(+)-3-ethyl-5-[5-(4-ethylpiperazin-1-ylsulphonyl)-2-(2-methoxy-1(R)-methylethoxy)pyridin-3-yl]-2-methyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one, also known as 3-ethyl-5-[5-[4-ethylpiperazin-1-ylsulphonyl]-2-[(1R)-2-methoxy-1-methylethyl]oxy)pyridin-3-yl]-2-methyl-2,6-dihydro-7H-pyrazolo[4,3-d] pyrimidin-7-one;

20

5-[2-ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-[2-methoxyethyl]-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one, also known as 1-{6-ethoxy-5-[3-ethyl-6,7-dihydro-2-(2-methoxyethyl)-7-oxo-2H-pyrazolo[4,3-d]pyrimidin-5-yl]-3-pyridylsulphonyl}-4-ethylpiperazine;

25

5-[2-*iso*-Butoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-(1-methylpiperidin-4-yl)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

5-[2-Ethoxy-5-(4-ethylpiperazin-1-ylsulphonyl)pyridin-3-yl]-3-ethyl-2-phenyl-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

30

5-(5-Acetyl-2-propoxy-3-pyridinyl)-3-ethyl-2-(1-isopropyl-3-azetidiny)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;

35

5-(5-Acetyl-2-butoxy-3-pyridinyl)-3-ethyl-2-(1-ethyl-3-azetidiny)-2,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one;



(6R,12aR)-2,3,6,7,12,12a-hexahydro-2-methyl-6-(3,4-methylenedioxyphenyl)pyrazino[2',1':6,1]pyrido[3,4-b]indole-1,4-dione (IC-351);

2-[2-ethoxy-5-(4-ethyl-piperazin-1-yl-1-sulphonyl)-phenyl]-5-methyl-7-propyl-3H-imidazo[5,1-f][1,2,4]triazin-4-one (vardeafil) also known as 1-[[3-(3,4-dihydro-5-methyl-4-oxo-7-propylimidazo[5,1-f]-as-triazin-2-yl)-4-ethoxyphenyl]sulphonyl]-4-ethylpiperazine; and

the compound of example 11 of published international application WO93/07124 (EISAI).

36. Use according to claim 35 wherein the PDE5i is sildenafil.

37. A pharmaceutical composition comprising a NEPi and a PDE5i for the treatment of MED.

38. A kit comprising a first component and a second component for the treatment of MED wherein the first component comprises a NEPi and wherein the second component comprises a PDE5i.

39. The kit of claim 38 wherein the NEPi is as defined in any of claims 1 to 29.

40. Use of compounds which act via inhibiting the mechanism that terminates the biological activity of a number of bioactive peptides and in particular vasoactive peptides, more particularly neuropeptides, that are released during sexual arousal for the treatment of MED.

41. Use of compounds which acts via enhancing a non-NO dependant NANC pathway for the treatment of MED.

ABSTRACT

- 5 The present invention relates to the use of selective neural endopeptidase inhibitors for the treatment of male sexual dysfunction, in particular MED.